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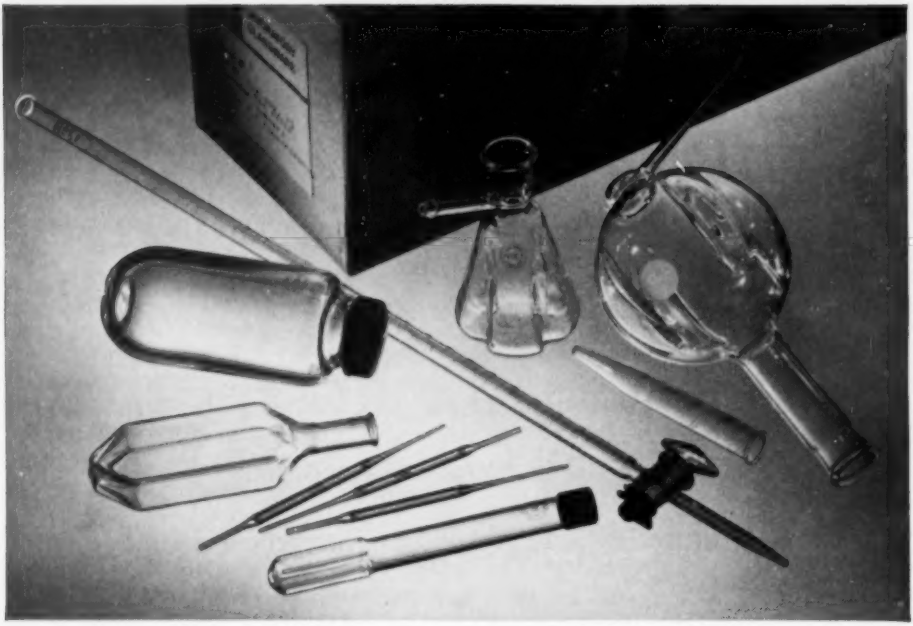

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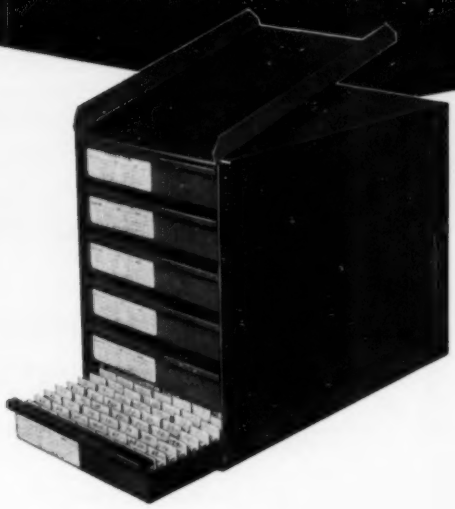

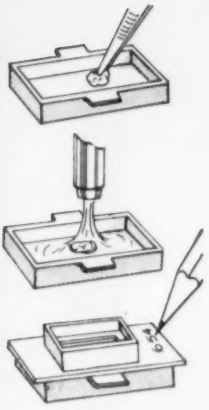

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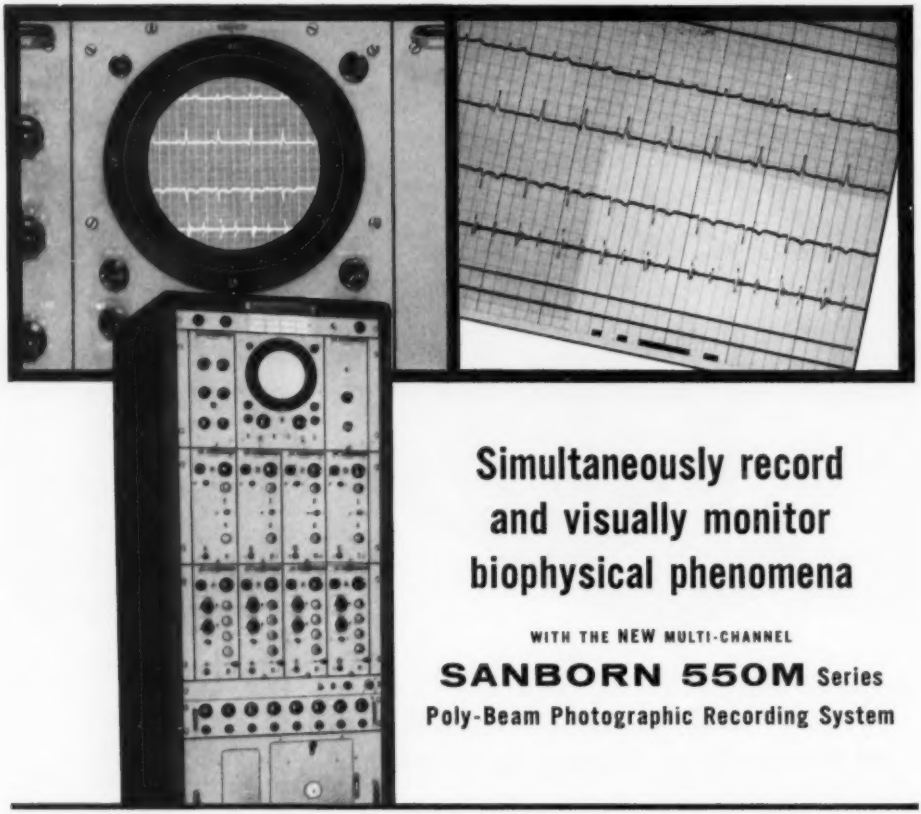

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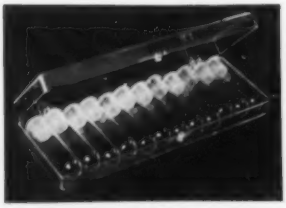

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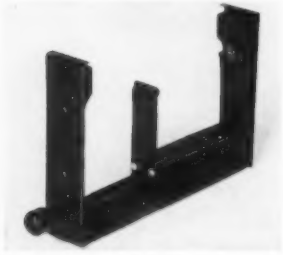

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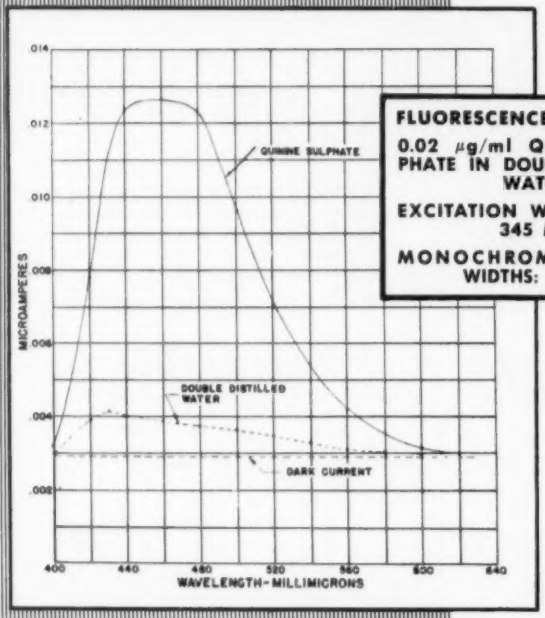

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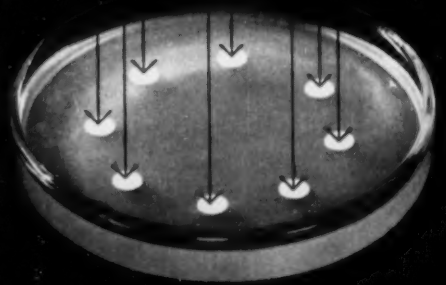

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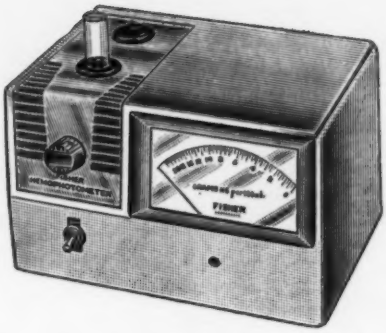

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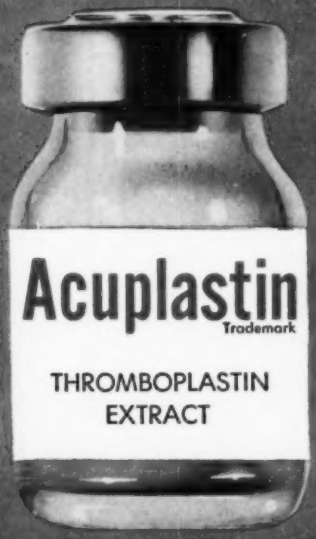

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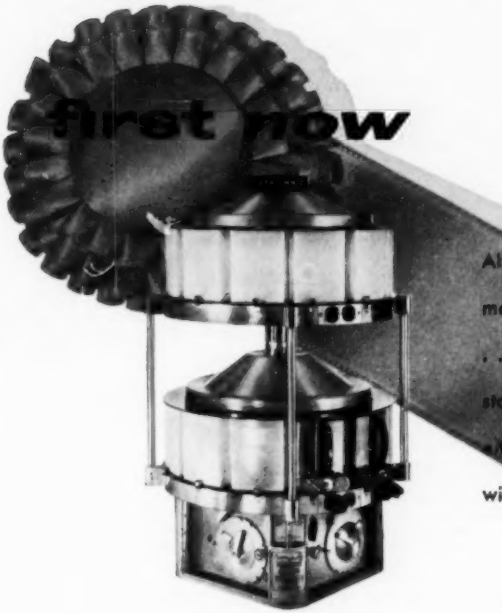

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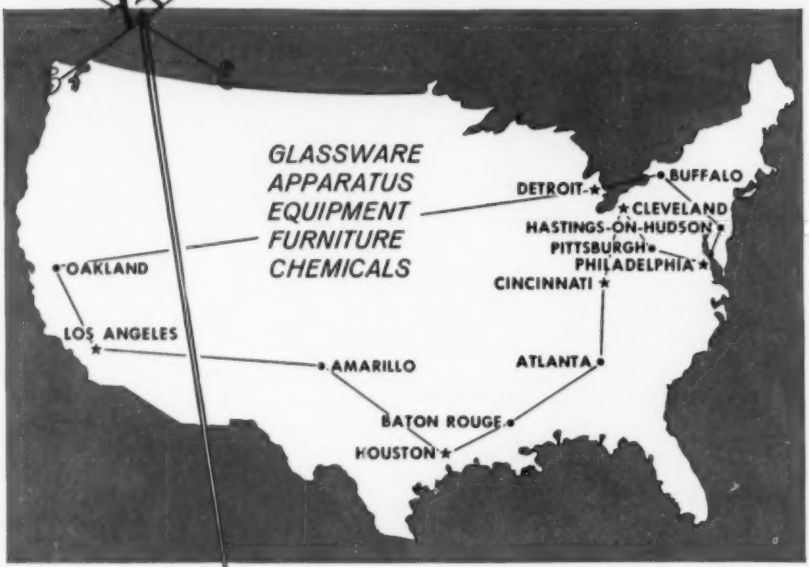

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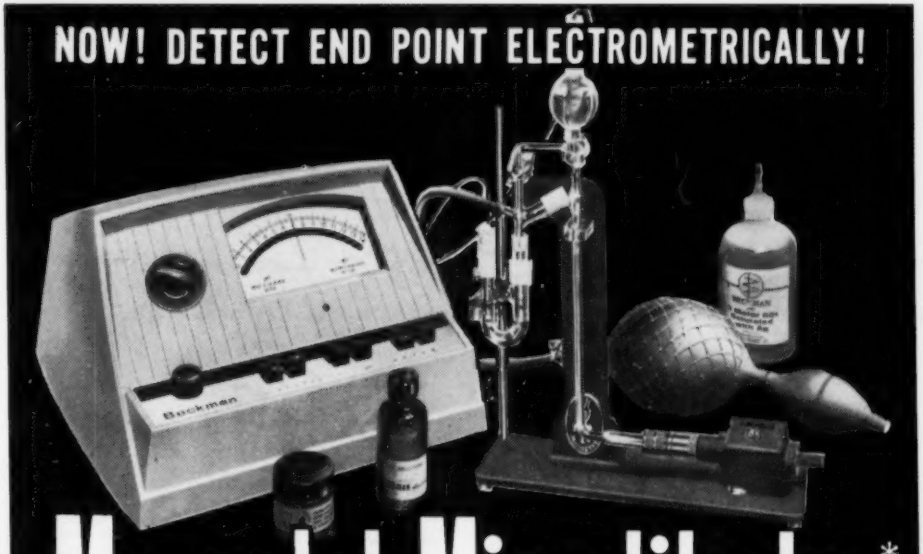

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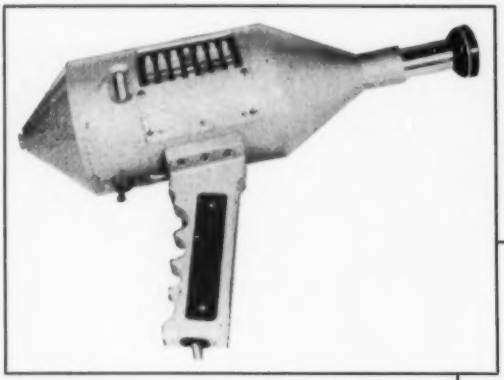

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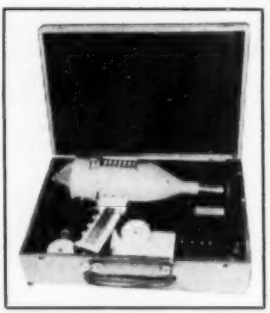

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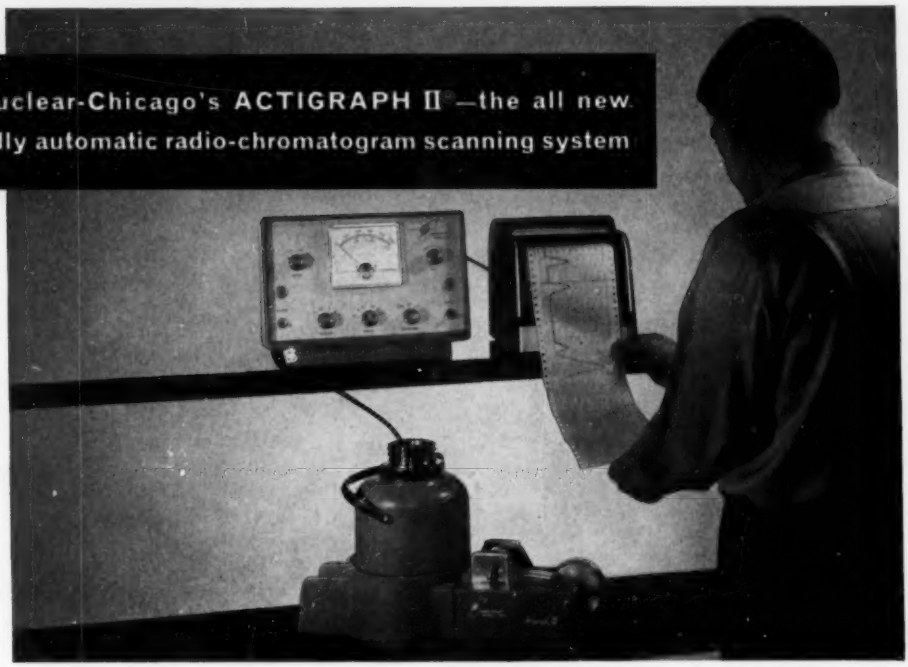

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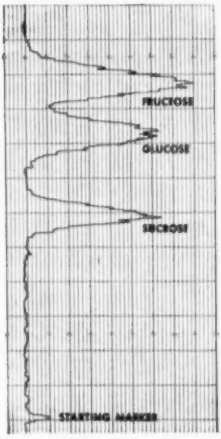

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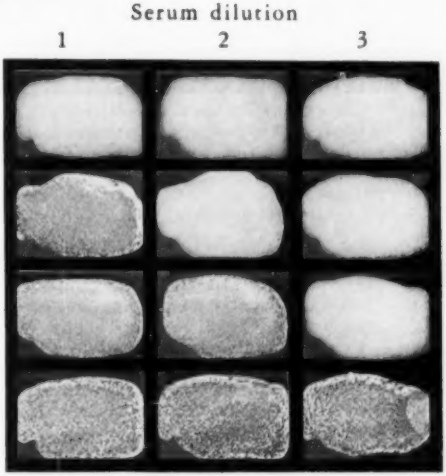

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VOL. 53 MARCH, 1959 No. 3

CLINICAL AND EXPERIMENTAL

THE MORPHOLOGIC BASIS OF ANTIBODY FORMATION DEVELOPMENT DURING THE NEONATAL PERIOD

ROBERT A. BRIDGES, M.D.,* RICHARD M. CONDIE, SOLOMON J. ZAK, M.D., AND ROBERT A. GOOD, M.D., PH.D.**
MINNEAPOLIS, MINN.

THE rapid somatic and physiologic maturation that occurs during the latter part of gestation and during the neonatal period has provided an unusual opportunity to gain an understanding of various biologic processes.[1] Our interest in the synthesis of gamma globulin and the cellular basis of antibody formation has led to a consideration of the development of these processes as revealed by changes occurring during the neonatal period. Although newborn mammals have long been considered to be poor antibody producers, the extent of this defect, and its morphologic or physiologic basis, has never been precisely defined. Osborn and associates[2] have presented data indicating that, during the second month of postnatal life, human infants begin to form circulating antibody. However, antigenic stimulation during the first month of life results in a quantitatively lesser response than that which occurs following such stimulation at a later time during the first year. A similar pattern of response has been demonstrated in various experimental animals. Freund[3] has shown, using sheep red blood cells and *Salmonella typhosa* as antigens, that while an occasional rabbit will produce antibody during the second week of life, the immune response in rabbits prior to 21 days of age is quite irregular.

From the Pediatric Research Laboratories of the Variety Club Heart Hospital, Minneapolis, Minn.

Aided by grants from the United States Public Health Service, the American Heart Association, the Minnesota Heart Association, and the Minnesota Division of the American Cancer Society.

Received for publication July 17, 1958.

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In human infants, the decline in the concentration of gamma globulin which regularly occurs during the first months of life[4-7] suggests either an excessive rate of catabolism or, as seems more likely, a deficient rate of synthesis. When this falling gamma globulin concentration is expressed in terms of half-life values, a striking similarity is observed between these decay rates and those found in children with congenital agammaglobulinemia following the parenteral administration of gamma globulin.[8] Between the second and fourth months of life there is, in the normal infant, a short period during which the decline in circulating gamma globulin concentration ceases and, as gamma globulin production develops, there begins a period of rising concentration that continues into the second year of life. At this time, gamma globulin values are attained which approximate those found in normal adults. During this later period of rising gamma globulin concentration, the infant is capable of adequate antibody production in response to antigenic stimulation.

Part I. Neonatal Agammaglobulinemia: Immunologic Studies of a Child Born of a Mother Suffering From Acquired Agammaglobinemia

We have recently had the opportunity to study, from birth, a child in whom the period of immunologic maturation was placed in sharp focus. This baby was a normal child born to a woman with acquired agammaglobulinemia. The observations resulting from this study have provided the basis for the other clinical and experimental investigations to be presented in this report. It is the purpose of this paper to describe the immunologic maturation and development of serum gamma globulin concentrations during the first year of life in the baby born to this mother and to present hematologic and pathologic studies carried out on normal newborn infants and children. In the last part of this report, we will present a series of experiments concerning the immunologic capacity of the rabbit during the neonatal period in terms of both the morphologic and antibody response. These several studies present a consistent correlation between the immunologic inadequacy of the neonate and certain morphologic deficiencies in the lymphoid and hematopoietic tissues characteristic of this period of life.

MATERIAL AND METHODS

A 33-year-old white woman suffering from acquired agammaglobulinemia has been under study in our laboratories since 1952. This patient, originally reported by Prasad and Koza,[9] has been shown to lack gamma globulin by paper and free electrophoretic analysis, to be of blood group O and to lack both anti-A and anti-B isoagglutinins. As with other agammaglobulinemic patients, intensive and repeated stimulation with a wide variety of potent bacterial and viral antigens did not result in the formation of circulating antibodies.[10-12] Hematologic studies revealed the virtual absence of plasma cells from her lymph nodes, bone marrow, and spleen despite intensive antigenic stimulation. However, during the last trimester of this pregnancy with the child to be described, antigenic stimulation with typhoid-paratyphoid vaccine resulted in a minimal but definite antibody response against the H, O, and B antigens, yet there had been no response to this same stimulation during the first two trimesters of pregnancy.

On Oct. 22, 1954, she gave birth to a normal female infant. Following delivery of the placenta and child, the antibodies present in the mother's blood gradually disappeared and subsequent antigenic stimulation revealed that the patient had again become incapable of an immune response to these same antigens. At no time during the pregnancy was

gamma globulin demonstrable by electrophoresis. Immunochemical determinations during pregnancy[13, 14] revealed that the actual concentration of gamma globulin remained consistently below 12 mg. per 100 ml. in spite of the slight immunologic response previously described.

During the neonatal period antigenic stimulation was provided to the child by the administration of TAB vaccine (Lederle) for immunization. One-tenth millimeter was given intradermally and 0.4 ml. was given subcutaneously at the same site. This vaccine contains 1,000 million *S. typhosa*, 250 million paratyphi A, and 250 million paratyphi B organisms per milliliter. This antigen was given initially on the second day of life and then at weekly intervals thereafter during the first 2 months of life. The same quantity of antigen was then given at monthly intervals throughout the remainder of the first year of life. Cord blood was obtained from the placenta immediately after delivery, and venous blood was obtained from the internal jugular vein at 2 and 7 days of age and then at weekly intervals during the first 2½ months and at monthly intervals thereafter until the baby was 14 months of age. A final specimen was obtained when the child was 2 years old. The determination of agglutinins against H, O, and B antigens was performed by serial twofold dilutions of serum in saline beginning with a 1:5 dilution. Three-tenths millimeter of these dilutions was mixed with 0.3 ml. of a standardized suspension of each antigen. The sealed tubes were incubated at 37° C. and the antibody titer read the following day after standing for 2 hours at 4° C. The end point was taken as the last tube which showed agglutination. Known positive and negative serum controls were included with each series of determinations. The H, O, and B antigens used in this study were generously provided by Dr. Henry Bauer of the Minnesota State Board of Health. The baby was found to be blood group O. Isoagglutinins against heterologous blood groups were performed after blood typing using commercial typing sera (Ortho) by making serial twofold dilutions of serum in saline starting with a 1:5 dilution. Fresh cells were obtained from appropriate donors and washed 3 times in 0.145 M saline or until the supernatant was clear. When negative results were obtained in the lowest dilution, full strength serum was used to exclude the presence of minute amounts of antibody. To 0.5 ml. of each serum dilution, 0.1 ml. of a 2 per cent suspension of washed cells was added. Saline controls were run with each series of determinations. These were mixed and then incubated in a water bath at 37° C. for 30 minutes. All tubes were then centrifuged at 1,000 r.p.m. for one minute in an International centrifuge (size 2, Model V). The last tube with agglutination of the red cells was taken as the titer.

Serum gamma globulin levels have been determined by an immunochemical method,[13, 14] and by analysis of total protein using a standard biuret technique together with paper electrophoresis or free moving boundary electrophoresis. The paper electrophoretic patterns were run on a Spinco Model E electrophoresis apparatus and analyzed on a Spinco Analytrol. Free electrophoresis was performed on a Klett free electrophoresis apparatus utilizing a veronal buffer of pH 8.6 and 0.1 ionic strength. Percentage values for each protein fraction were then calculated and the gamma globulin fraction determined in reference to the total protein determination. The zinc turbidity determinations of gamma globulin were based on the method of Kunkel.[15] Lymph node biopsies were obtained from the baby in the immediate neonatal period, at 6 days of age (4 days following local antigenic stimulation), at 3 months of age, and again at 14 months of age. Imprints of the node were prepared and stained with Wright-Giemsa by the method of Downey. In addition, material from the lymph node biopsy was fixed with formol saline or absolute alcohol and stained with hematoxylin-eosin or methyl green-pyronine according to standard procedures.

RESULTS

During the neonatal period this child was clinically indistinguishable, by all morphologic and immunologic criteria, from a patient with agammaglobulinemia.[8] For example, as shown in Fig. 1, the paper and free electrophoretic

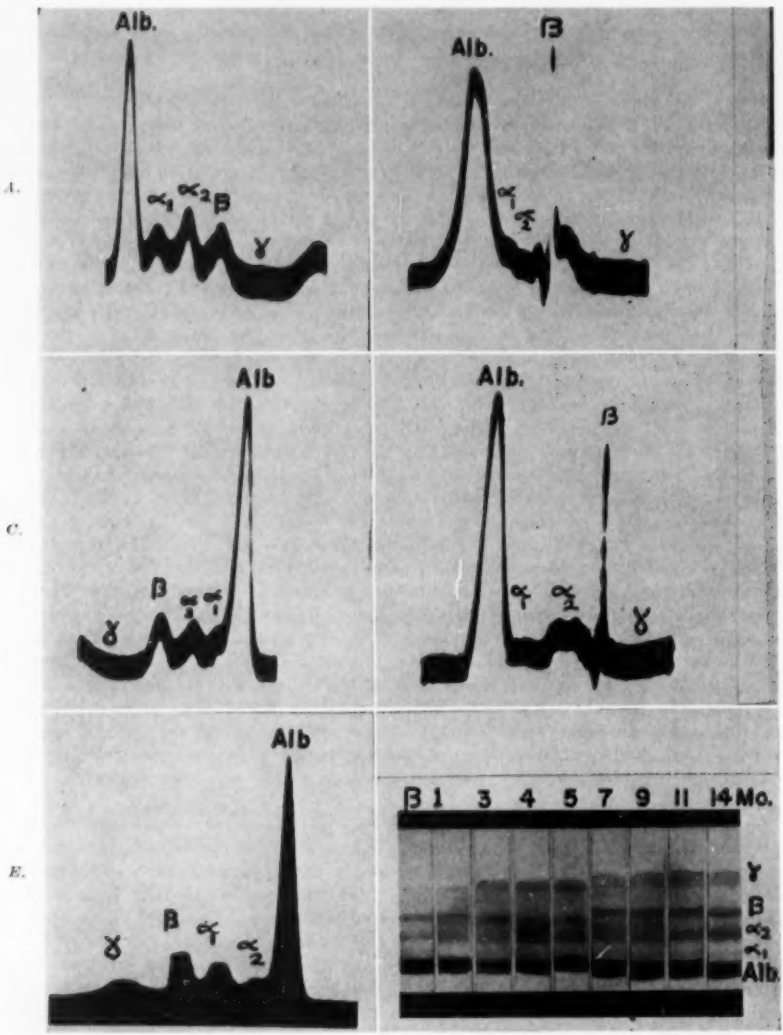

Fig. 1.—Electrophoretic patterns during the first year of life in immunologically normal child born of an agammaglobulinemic mother. *A*, Ascending boundary electrophoretic pattern of the mother's serum taken at time of delivery of the child. *B*, Descending boundary of maternal electrophoretic pattern. *C*, Ascending boundary of the infant's electrophoretic pattern of serum obtained by venepuncture at 2 days of age. Note complete absence of gamma globulin peak. *D*, Descending boundary of electrophoretic pattern obtained from baby at 2 months of age. Gamma globulin is still absent from the serum. *E*, Ascending boundary of electrophoretic pattern obtained from serum of the baby at 14 months of age. Gamma globulin is now present in normal amounts. *F*, Serial paper electrophoretic patterns obtained on the baby born of agammaglobulinemic mother. This picture illustrates the growth of gamma globulin synthesis during the first year of life. At birth, gamma globulin is completely absent. Gamma globulin appears at 3 months and increases rapidly and steadily in amount during the remainder of the first year of life.

patterns of both the mother and baby lacked a gamma globulin peak. By immunologic determination, the gamma globulin concentration in both the cord and maternal serum was 11 mg. per 100 ml. Plasma cells were absent from the bone marrow of both the mother and the baby (Table I). The lymph node

TABLE I. COMPARISON OF NUMBERS OF PLASMA CELLS IN BONE MARROW OF AGAMMAGLOBULINEMIC MOTHER, HER NEWBORN BABY (AN IMMUNOLOGICALLY MATURE CHILD), AND A PATIENT WITH CONGENITAL AGAMMAGLOBULINEMIA

PATIENT	AGE	NO. OF PLASMA CELLS PER 20,000 NUCLEATED MARROW CELLS	ANTI-O AGGLUTININ TITER—MAXIMUM TITER OBTAINED*	BLOOD GROUP	ISOAGGLUTININ ANTI-A	ISOAGGLUTININ ANTI-B
L. L. Pregnant, acquired agammaglobulinemia, prior to antigenic stimulation†	33 yr.	2	0	O	0	0
L. L. As above, 5 days following injection of antigen (third trimester)	33 yr.	1	1/80*	O	0	0
R.L. Newborn female, born of agammaglobulinemic mother	2 days	0	0	O	0	0
R. L. As above, + 4 days following injection of TAB vaccine	6 days	0	0	O	0	0
R. L. As above, 4 days following antigenic stimulation	3 mo.	23	1/160	O	1/10	0
R. L. As above, 4 days following antigenic stimulation	14 mo.	92	1/1,280	O	1/1,280	1/320
E. S. Ten-year-old with congenital agammaglobulinemia	10 yr.	0	0	O	0	0
E. S. As above, 4 days after antigenic stimulation	10 yr.	0	0	O	0	0
T. M. Normal, mentally deficient child	8 yr.	72	0	O	1/320	1/80
T. M. As above, 5 days following TAB vaccine	8 yr.	138	1/640†	O	1/320	1/80

*Maximum agglutinin titer obtained 14 days following intradermal injection of 0.1 ml. and subcutaneous injection of 0.4 ml. of Lederle TAB vaccine containing 1,000 million heat-killed *S. typhosa*, 250 million *S. paratyphi*, and 250 *S. schottmülleri* organisms per milliliter.

†Patient had no gamma globulin by electrophoretic method, but had 11 mg. per 100 ml. of serum by immunochemical measurement.

tissue of the baby at the time of biopsy on the second day of life was devoid of plasma cells, and the latter did not appear in the medullary portion of the lymph node on the fourth day following antigenic stimulation. During the first 2 months of life, weekly injections of typhoid-paratyphoid antigens did not result in the development of a demonstrable amount of circulating antibody. The serum gamma globulin concentration remained at or below 11 mg. per 100 ml. during the first month of the baby's life and did not show a concentration higher than this base-line level until the forty-second day when the concentration

was found to be 115 mg. per 100 ml. Following this initial rise, the gamma globulin concentration in the baby's serum rose rapidly to levels normally observed during the first year of life. By paper electrophoresis, gamma globulin was not detectable until the sixty-seventh day of life. At this same time, on the sixty-seventh day, a titer of 1:160 against the H antigen of the TAB vaccine was first observed. At 3 months of age another lymph node biopsy was performed in the area draining the site of an intracutaneous and subcutaneous injection of typhoid-paratyphoid vaccine. The lymph node at this time possessed a morphologic appearance more like that found in adult lymph node tissue. Both primary and secondary nodules were present* and plasma cells were scattered throughout the medullary cords.

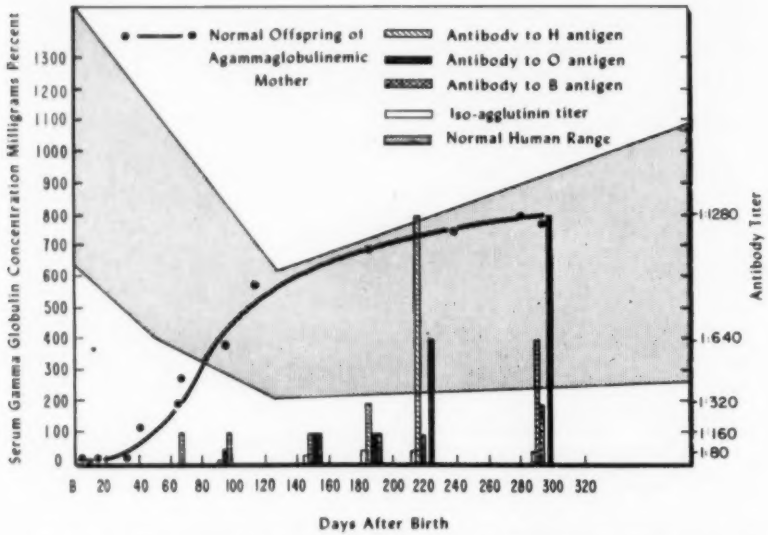

Fig. 2.—The development of gamma globulin and the formation of circulating antibody in the baby born to a woman with acquired agammaglobulinemia. The details concerning antigenic stimulation are given in the text. The shaded area over the graph indicates the normal range of gamma globulin concentration during this period of life as determined by the immunochemical technique in our laboratory. The solid line plots the gamma globulin concentration present in the baby studied while the vertical bars represent the titers of the various antibodies measured as indicated in the legends on the graph.

By 3 months of age, circulating agglutinins against the H, O, and B antigens of the TAB vaccine were present. In this baby (blood group O), isoagglutinins against both A and B cells were lacking at birth and throughout the first 2 months of life. At 3 months of age, however, isoagglutinins in a titer of 1:10 were present against A cells although the anti-B titer was still negative. By the sixth month the anti-A titer had risen to 1:80 and the anti-B titer to 1:10.

*The terminology used in this paper in reference to nodular formations within the lymph nodes is defined as follows. This is modified after Gyllensten.[10] *Large primary nodule:* The diffuse collection of cortical lymphocytes bounded by the cortical trabeculae and the medullary cords. *Small primary nodule:* The dense collections of small lymphocytes found within the cortical tissue. *Secondary nodule:* Small primary that have formed centers. The center activity may be further described as early, high active, or terminal.[11]

The cases of addition of bacteriostatic and bactericidal activity with the 38 paired antibiotic combinations (agar diffusion method with replica plating) are given in Table III. Additive potentiation of the bacteriostatic activity of oleandomycin and its mixtures appeared most frequently with bacitracin, chloramphenicol, erythromycin and, as might be expected, by combinations of pairs within the oleandomycin mixtures. From these results it would appear that certain antibiotic combinations might be more effective than those presently available. Furthermore, different proportions of antibiotics in these mixtures might enhance their activity against staphylococci. The summation of bactericidal activity (Table III) showed a similar pattern, with neomycin appearing more effective and tetracycline less effective than in bacteriostasis.

TABLE III. ADDITIVE ANTIBACTERIAL ACTIVITY OF 12 ANTIBIOTICS WITH 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (BY AGAR DIFFUSION WITH REPLICA PLATING)

	BACTERIOSTATIC COMBINATIONS (ORIGINAL PLATE)				BACTERICIDAL COMBINATIONS (REPLICA PLATE)				
ANTIBIOTIC	O	AP	APG	Si	Si	APG	AP	O	
AP	24							18	AP
APG	23	16					18	17	APG
Si	20	20	12			12	12	24	Si
B	28	26	24	25	24	22	29	24	B
C	22	20	21	23	18	20	23	24	N
E	21	15	18	21	20	14	16	23	C
No	17	15	15	12	21	11	13	20	E
T	12	12	11	11	13	14	13	14	P
P	11	10	14	11	10	12	16	15	No
N	8	8	7	5	10	6	12	10	T
V	1	5	3	2	4	7	10	5	V

Key to abbreviations: AP = oleandomycin-penicillin; APG = oleandomycin-penicillin G; Si = Sigmamycin (oleandomycin-tetracycline); O = oleandomycin; B = bacitracin; C = chloramphenicol; E = erythromycin; No = novobiocin; T = tetracycline; P = penicillin; N = neomycin; V = vancomycin. The number given indicates the number of strains out of 30 showing addition of antibacterial activity with a pair of antibiotics.

No instance of true synergism was found in any combination of 8 common antibiotics with oleandomycin or its mixtures. Antagonism of bacteriostatic activity was exceedingly rare with oleandomycin combinations (Table IV). As seen in Table V, the 3 cases of antagonism of bacteriostasis all occurred with just one strain of staphylococcus (No. 10). Antagonism of bactericidal activity as shown in Table IV, although not frequent, appeared more often. Antagonism of bactericidal effects occurred in 79 cases, including only 18 out of the 30 strains of staphylococcus. Nine strains showed antagonism of bactericidal activity with 3 or more antibiotic combinations. One-third of these strains accounted for 82 per cent of the cases of antagonism. It should be noted that 38 antibiotic combinations were tested against 30 strains of staphylococcus; therefore, Table IV represents the incidence of antagonism in 1,140 possible cases.

The combined antibacterial effects, both bacteriostatic and bactericidal, with each of the 30 strains of staphylococcus and 38 paired antibiotic combinations containing oleandomycin are shown in Table V. There were 5

TABLE IV. ANTAGONISTIC ANTIBACTERIAL ACTIVITY OF 12 ANTIBIOTICS WITH 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (BY AGAR DIFFUSION WITH REPLICA PLATING)

	BACTERIOSTATIC COMBINATIONS (ORIGINAL PLATE)				BACTERICIDAL COMBINATIONS (REPLICA PLATE)				
ANTIBIOTIC	O	Si	APG	AP	AP	APG	Si	O	
Si	0							0	Si
APG	0	0					5	5	APG
AP	0	0	0			0	7	7	AP
No	0	0	0	0	0	3	5	4	No
E	0	0	1	1	7	5	0	0	E
V	0	0	0	0	0	0	0	8	V
T	0	1	0	0	3	4	0	0	T
P	0	0	0	0	0	0	3	3	P
C	0	0	0	0	0	5	0	0	C
N	0	0	0	0	0	0	0	0	N
B	0	0	0	0	0	0	0	0	B

Note: For key to abbreviations see Table III.

The number given indicates the number of strains out of 30 showing antagonism of antibacterial activity with a pair of antibiotics.

TABLE V. COMBINED ANTIBACTERIAL EFFECTS OF 38 OLEANDOMYCIN COMBINATIONS AGAINST 30 HOSPITAL STRAINS OF STAPHYLOCOCCUS AUREUS (BY AGAR DIFFUSION WITH REPLICA PLATING)

	NO EFFECT		ADDITION		ANTAGONISM	
STRAIN	ORIGINAL PLATE	REPLICA PLATE	ORIGINAL PLATE	REPLICA PLATE	ORIGINAL PLATE	REPLICA PLATE
1	17*	14	21	17	0	7
2	17	22	21	21	0	12
3	22	17	16	26	0	0
4	23	17	15	19	0	2
5	21	17	17	20	0	1
6	21	12	17	25	0	1
7	19	22	19	15	0	1
8	22	18	16	18	0	2
9	14	13	24	21	0	4
10	16	25	19	13	3	0
11†	29	29	9	9	0	0
12	11	11	27	27	0	0
13†	25	28	13	10	0	0
14	11	14	27	19	0	5
15†	29	28	9	10	0	0
16	24	16	14	20	0	2
17†	25	25	13	13	0	0
18	25	13	13	17	0	8
19	9	11	29	27	0	0
20	19	13	19	25	0	0
21	20	16	18	20	0	2
22†	30	31	8	7	0	0
23	15	4	23	23	0	11
24	11	6	27	31	0	1
25	14	9	24	29	0	0
26	19	17	19	19	0	2
27	16	10	22	17	0	11
28	14	9	24	24	0	5
29	18	12	20	25	0	1
30	11	11	27	27	0	0

*Each number refers to the number of cases of combined effect out of a possible total of 38.

†These strains were highly resistant by the tube method to oleandomycin, AP, APG, Sigmamycin, and many of the other antibiotics tested. It will be noted that none of these strains showed antagonism and all showed less addition than the more susceptible strains. Other strains showing less resistance had a higher incidence of addition and antagonism. This demonstrates that for an organism to react favorably or unfavorably to a combination it must be susceptible to the antibiotics used. No instances of synergism were found.

strains of staphylococcus (Nos. 11, 13, 15, 17, and 22) highly resistant to the oleandomycin group, as well as to other antibiotics. The resistant strains, as might be expected, showed less addition of antibiotic activity than the more susceptible strains. Furthermore, the highly resistant strains showed no antagonism between antibiotics. Three susceptible strains (Nos. 2, 23, and 27) showed a higher incidence of antagonism. Table VI again demonstrates that resistant strains of staphylococcus showed less addition of antibiotic activity than did the susceptible strains. Likewise, resistant strains showed less antagonism of antibiotic bactericidal effects.

TABLE VI. ANTIBACTERIAL ACTIVITY OF 4 OLEANDOMYCIN COMBINATIONS AND 8 OTHER ANTIBIOTICS IN PAIRS AGAINST RESISTANT AND SUSCEPTIBLE STRAINS OF STAPHYLOCOCCUS AUREUS

ANTIBIOTIC	NUMBER OF STRAINS	ORIGINAL PLATE			REPLICA PLATE		
		NO EFFECT (PER CENT)	ADDITION (PER CENT)	ANTAGONISM (PER CENT)	NO EFFECT (PER CENT)	ADDITION (PER CENT)	ANTAGONISM (PER CENT)
*A. Resistant**							
Oleandomycin	5	60†	40	0	58	42	0
Sigamycin	7	81	19	0	77	18	5
AP	5	71	29	0	71	29	0
APG	5	69	31	0	78	22	0
B. Susceptible							
Oleandomycin	25	40	60	0	28	62.2	9.8
Sigamycin	23	41.5	58.1	0.4	32.4	61.3	6.3
AP	25	43.6	56	0.4	29.8	60	10.2
APG	25	46.2	53.4	0.4	38.9	51.3	9.8

*Resistant to 6.25 μg or more per milliliter of antibiotic in column 1, by the tube dilution method.

†Each number represents the per cent of cases showing addition, antagonism, or no effect upon antibacterial activity when tested in combination with another antibiotic.

TABLE VII. EFFECT OF ANTIBIOTIC RESISTANCE UPON ACTIVITY OF OLEANDOMYCIN COMBINATIONS AGAINST HOSPITAL STAPHYLOCOCCI

ANTIBIOTIC	NUMBER OF STRAINS	ORIGINAL PLATE		REPLICA PLATE	
		NO EFFECT (PER CENT)	ADDITIVE (PER CENT)	NO EFFECT (PER CENT)	ADDITIVE (PER CENT)
1. Sigmamycin Tetracycline					
Susceptible	10	0	100	10	90
Resistant	20	90	10	90	10
2. Oleandopen (AP) Penicillin G					
Susceptible	10	50	50	30	70
Resistant	20	75	25	70	30
3. Fortified oleandopen (APG) Penicillin G					
Susceptible	10	20	80	30	70
Resistant	20	70	30	65	35

Note: None of these combinations proved antagonistic or synergistic.

In Table VII it is shown that oleandomycin had enhanced bacteriostatic and bactericidal effects against susceptible staphylococci upon the addition of tetracycline or penicillin G. Additive potentiation was rarely seen with

resistant strains. This confirms earlier reports[6-10] that antibiotics in pairs have little or no combined effect on bacterial growth if the strain of staphylococcus is resistant to either antibiotic.

DISCUSSION

The present studies confirm the fact[6-8] that erythromycin is much more effective than the closely related antibiotic, oleandomycin, in retarding the multiplication of staphylococci. Jones and Finland[6] have also shown that erythromycin produces greater antistaphylococcal activity in the blood of human beings after a single oral dose. Erythromycin was far more active than any of the oleandomycin combinations, particularly if subcultures of broth tubes were examined for bactericidal effect. Even in high concentrations oleandomycin failed to kill most strains of hospital staphylococcus.

Sigmamycin, a mixture of one part oleandomycin and two parts tetracycline, was more active than either component alone. The potentiation measured by agar diffusion was additive rather than truly synergistic, as claimed by English and associates.[1] Furthermore, potentiation was seldom seen with strains of staphylococcus resistant to tetracycline, which are now frequent in hospitals.

The oleandomycin salt of penicillin G (oleandopen or AP) was more actively bacteriostatic than either oleandomycin or penicillin alone. Again the potentiation was additive in behavior rather than truly synergistic. Similar results were obtained with oleandopen fortified with additional penicillin G (APG). In neither case was there enhanced activity against penicillin-resistant strains.

Observations on cross-resistance to erythromycin and oleandomycin in hospital staphylococci in general tend to confirm the greater activity of erythromycin. One strain of staphylococcus which was naturally erythromycin-resistant, however, was susceptible to oleandomycin. Five strains resistant to oleandomycin were inhibited by erythromycin. Two strains in which erythromycin resistance was induced became much more resistant to oleandomycin. Yet after induction of resistance to oleandomycin, erythromycin resistance appeared in only one of the two strains. Others[10, 11] found that strains in which oleandomycin resistance was induced were regularly cross-resistant to erythromycin. Garrod[8] showed, on the contrary, that staphylococci naturally resistant to erythromycin, although often highly resistant to oleandomycin, spiramycin, and other antibiotics of the erythromycin group, may in some cases be resistant only to erythromycin. With the latter type of "dissociated" strains, Garrod[8] demonstrated that erythromycin in moderate concentrations permitted the staphylococci to grow in the presence of oleandomycin. The dissociated strains were not dependent upon erythromycin but growth stimulation was dependent upon the concentration of erythromycin in oleandomycin media.

The present work demonstrated neither antagonism nor synergism of action between erythromycin and oleandomycin against either susceptible or

resistant strains of staphylococcus. These two antibiotics frequently had additive antibacterial activity but with resistant strains only the most active antibiotic was effective, and this usually was erythromycin.

Potentiation of the bacteriostatic effects of oleandomycin was seen much more frequently upon the addition of bacitracin, chloramphenicol, and erythromycin than with tetracycline in these resistant hospital strains of staphylococcus. This is to be expected. It is pertinent that of 179 strains of coagulase-positive staphylococcus isolated from the blood or infected lesions of patients in this hospital in 1957, the percentage resistant to various antibiotics (disk method) was as follows: tetracycline 69, penicillin 68, streptomycin 66, erythromycin 28, chloramphenicol 18, novobiocin 1, and neomycin 0.6.

Previous studies[9] with the same 30 hospital strains used in the present work showed antagonism of the bactericidal action of novobiocin by several commonly used antibiotics. The present investigations revealed antagonism of this type most frequently with Sigmamycin, especially in combination with oleandopen (AP), fortified oleandopen (APG), and novobiocin. Antagonism of bactericidal activity was fairly frequent with oleandomycin combinations, particularly with vancomycin and oleandopen (AP). Antagonism with erythromycin combinations was much less frequent.[9]

Antagonism of antibacterial effects occurred only with susceptible strains of staphylococcus (Table VI) and seemed to be characteristic of certain of these cultures (Table V). Additive potentiation of antibiotic effects was seen frequently with susceptible strains but rarely with resistant strains (Tables V, VI, and VII). This observation does not support the conclusion of English and his group[1] that synergistic potentiation occurs with Sigmamycin against resistant staphylococci. It may be important that they used selected strains of staphylococcus and their broth method of in vitro measurement permits a considerable variation in results when repeated or compared with results obtained by agar diffusion.

SUMMARY AND CONCLUSIONS

1. Thirty hospital strains of coagulase-positive, hemolytic *Staphylococcus aureus* were exposed to individual antibiotics of the erythromycin group: erythromycin, oleandomycin, oleandopen (AP), fortified oleandopen (APG), and Sigmamycin. These strains were then tested against pairs of all of these antibiotics and bacitracin, chloramphenicol, tetracycline, penicillin, neomycin, vancomycin, and novobiocin. The tube dilution technique was used to measure bacteriostasis by single antibiotics and subculture of the clear tubes to measure bactericidal end points. The paper strip-agar diffusion method was used to test the bacteriostatic effect of antibiotic pairs. Replica plates were prepared to record the bactericidal action of antibiotic pairs.

2. The most active drugs as measured by bacteriostatic effect were erythromycin and Sigmamycin. Sigmamycin was more active than either of its two components, oleandomycin and tetracycline. The oleandomycin-penicillin mixtures (AP and APG) were slightly more active than oleandomycin and much more active than penicillin G against these predominantly penicillin-resistant strains.

3. Tests of the bactericidal action showed erythromycin to be the most active drug in the erythromycin group. Oleandomycin was the least active of the antibiotics tested.

4. Cross-resistance between erythromycin and oleandomycin was frequent in erythromycin-resistant staphylococci but the reverse situation was not frequent.

5. Additive potentiation of the bacteriostatic activity of oleandomycin and its mixtures in paired combinations with other antibiotics (agar diffusion method) appeared most frequently with bacitracin, chloramphenicol, erythromycin, and, as expected, with other oleandomycin mixtures. Potentiation of the bactericidal activity (replica plating method) was not frequent with neomycin. Tetracycline was less effective in increasing bactericidal action than in enhancing bacteriostasis.

6. No instances of true synergism of antibacterial effects were seen with any of the antibiotic combinations studied.

7. Antagonism of antibiotic bacteriostasis was rare. Antagonism of bactericidal activity occurred more frequently, particularly with certain susceptible strains. One-third of the strains accounted for 82 per cent of the instances of antagonism.

8. Most of the antibiotic interaction, both addition and antagonism, occurred with the more susceptible strains of staphylococcus.

Technical assistance in the experiments on cross-resistance to erythromycin and oleandomycin was given by Miss Marilyn J. Johnson.

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PANCREATIC ACTION OF THE SULFONYLUREAS

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THE sulfonylurea compounds are now widely employed in the treatment of diabetes mellitus. Their exact influence on carbohydrate metabolism has not yet been elucidated. Information concerning the means by which these agents lower the blood glucose may shed light on the mechanism of pancreatic insulin secretion and the nature of diabetes itself.

Loubatières and associates[1] in 1942 began their studies of the hypoglycemic action of the isopropylthiodiazole derivative of sulfanilamide and similar drugs, and in 1946 suggested its use in diabetic patients.[2] In the last 3 years interest in this problem has revived, and a wealth of experimental data has accumulated. Many of these articles present only preliminary results or conflicting information. Stetten[3] has recently pointed out certain inconsistencies of the studies reported thus far, and called for a re-evaluation of the problem. It is the purpose of this paper to present direct evidence regarding the site of action of these drugs, and to review critically the pertinent literature.

Earlier experiments have been described elsewhere.[4-6] Acute operative studies have been performed on 59 dogs to determine the consequences of perfusing sulfonylurea solutions through the pancreas and liver. The data support the general consensus that these compounds cause hypoglycemia primarily by their influence on the pancreas. A stimulation of insulin release by the beta cells of the islets of Langerhans is postulated. The exact mode of action of the sulfonylureas may remain unknown until a better understanding is gained of the means of synthesis, storage, and secretion of insulin.

METHODS

Animals.—Small amounts of tolbutamide,* carbutamide,† or control substances were introduced into the pancreatic artery in 27, into the femoral vein in 17, into the hepatic artery in 8, and into the portal vein in 7 dogs. Healthy mongrel animals of both sexes weighing between 10 and 25 kilograms consumed a ration containing approximately 40 per cent carbohydrate, 35 per cent protein, and 25 per cent fat ad libitum for one week prior to the experiment. They were fasted for 18 hours immediately before operation.

Surgical Procedure.—Animals were anesthetized intravenously by means of 25 mg. of Nembutal for each kilogram of body weight. They were maintained in a light plane of

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*Orinase (or 1-butyl-3-p-tolylsulfonylurea); The Upjohn Company, Kalamazoo, Mich.
†BZ-55 (or 1-butyl-3-sulfonylurea); Eli Lilly & Company, Indianapolis, Ind.

anesthesia with small subsequent doses. Total amounts given over the experimental period ranged from 250 to 925 mg.

In dogs subjected to pancreatic perfusion, the right gastroepiploic artery was isolated and cannulated with a polyethylene catheter (Fig. 1). The pancreaticoduodenal artery was identified at its entrance into the pancreas. The test solution was injected at a slow rate in a retrograde direction by means of a constant infusion pump. In this fashion all the body and portions of the head and tail of the pancreas could be perfused without disturbing their blood supply. Anatomic dissections following dye injection have indicated that approximately one third to one half of the pancreas is included by this method (Fig. 2). Animals of the femoral vein group were subjected to dissection of the pancreatic circulation in order to reproduce the surgical stress obtained in the experimental dogs. Pancreatic ducts were ligated in 4 animals, and 6 months later the pancreatic artery injection was performed in the manner described above.

Another series of dogs received perfusion of the liver via the hepatic artery or portal vein. In the former technique, the left branch of the common hepatic artery was catheterized (Fig. 1) so that at least half of the organ could be perfused without completely interrupting the arterial supply. Portal injection was accomplished by cannulating a splenic or mesenteric tributary.

Perfusions.—Solutions containing 5 to 10 mg. per milliliter of tolbutamide (sodium salt) or carbutamide were adjusted to pH 7.2. Control solutions were identical in osmolarity and pH to experimental solutions but the sulfonylurea was omitted. In two animals of the pancreatic control group, sodium sulfadiazine instead of sulfonylurea was employed in the same dosage. Rate of perfusion varied from 0.4 to 0.9 ml. per minute and total volume from 7.0 to 19.3 ml. Duration of perfusion was 20 minutes in all animals except those treated for 90 minutes in a few early screening experiments. Total amount of sulfonylurea administered over these periods varied between 0.07 and 1.13 Gm. Dosage of tolbutamide or carbutamide was 1 to 71 mg. per kilogram of body weight. Table I illustrates that the mean dosage, volume, and perfusion rate of sulfonylurea were closely comparable for the experimental and control groups of the different injection sites.

Samples.—Blood specimens were withdrawn from the femoral vein immediately prior to anesthesia, operation, and perfusion and at intervals of 10 to 30 minutes following the beginning of the perfusion for 4 hours. Glucose was estimated by the method of Somogyi-Nelson.[7] Free blood sulfonamide was determined in the carbutamide-injected animals by the method of Bratton and Marshall.[8] Estimate of blood concentration was not accomplished in the tolbutamide series. In the carbutamide groups, biopsies were taken from the mid-portion of the head of the pancreas before and after perfusion, fixed in Bouin's solution and 70 per cent alcohol, and stained with aldehyde and fuchsin to demonstrate islet cells. Frozen pancreas from some animals was assayed for insulin.[9]

RESULTS

Appraisal of Experimental Procedure.—To justify comparison between the results of injection of various experimental or control solutions into the different sites, animals must be similar in physical condition, treated in the same fashion, and show a similar response to operative stress prior to perfusion. Table I gives values for these factors for groups of animals subjected to the different surgical procedures. It is seen that for the different sites in both the sulfonylurea and control groups, the mean body weight (17.0 to 19.9 Kg.), amount of anesthesia (33.3 to 41.0 mg. per kilogram of body weight), and duration of surgery before perfusion (86 to 120 minutes) were comparable.

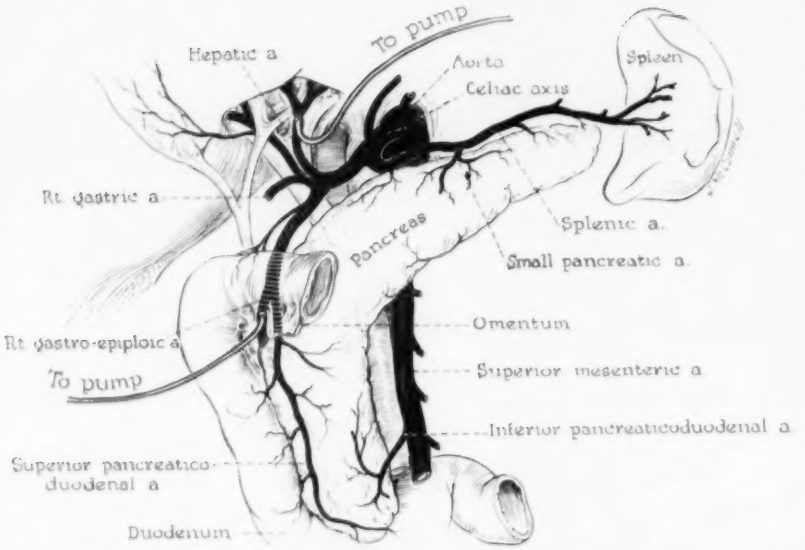

Fig. 1.—Arterial supply of the dog pancreas and liver. A polyethylene catheter has been placed in the right gastroepiploic artery so that pancreatic perfusion can be accomplished without interruption of the blood flow through the pancreaticoduodenal artery. Another catheter in the left hepatic artery permits perfusion of the liver.

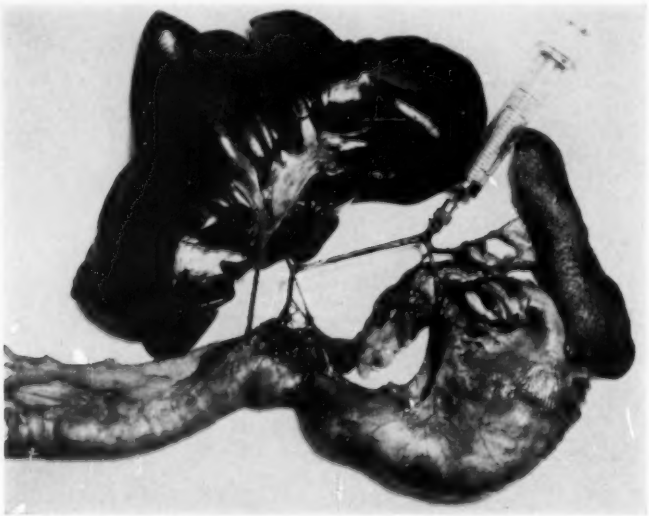

Fig. 2.—Anatomic preparation of the dog pancreas and liver, showing the arterial circulation (Fig. 1) outlined by methylene blue dye injection. An experimental solution introduced into the celiac axis is carried into the body and portions of the head and tail of the pancreas.

TABLE I. EXPERIMENTAL DATA REGARDING OPERATIVE TECHNIQUE IN EXPERIMENTAL AND CONTROL DOGS

SITE OF PERFUSION	AGENT	NO. OF DOGS	AVERAGE WEIGHT (KG.)	NEMBUTAL (MG./KG. B.W.)	AV. DURATION OF SURGERY (MIN.)	TOTAL DOSE OF SULFONYLUREA (GM.)	VOLUME (ML.)	RATE OF PERFUSION (ML./MIN.)
Pancreatic artery	Tolbutamide	6	18.6	39.3	91	0.13	12.9	0.65
	Control	4	18.6	41.0	86	--	13.0	0.65
Femoral vein	Tolbutamide	6	16.8	35.0	89	0.12	12.7	0.63
	Control	4	19.9	41.0	88	--	14.0	0.70
Hepatic artery	Tolbutamide	5	17.0	36.3	95	0.12	12.7	0.63
	Control	3	16.4	40.5	96	--	11.4	0.57
Portal vein	Carbutamide	3	16.5	35.0	132	0.12	11.6	0.55
	Control	4	14.4	33.3	114	--	10.0	0.51
Pancreatic artery (pancreatic ducts ligated)	Tolbutamide	4	17.7	41.0	120	0.12	14.2	0.71
Mean	(39 dogs)		17.3	38.0	101	(0.12)	12.6	0.62

Anesthesia caused a rise in mean blood glucose which was similar for all sites of injection.* Further increment in blood glucose during operation was almost the same for the 3 major sites.† This latter rise was also comparable in the sulfonylurea and nonsulfonylurea groups for each site (Figs. 3, 4, and 5). Dissection for cannulation of the portal vein, however, caused a 44 per cent elevation in blood glucose. Dogs in which the pancreatic ducts had previously been ligated showed 31 per cent rise in glucose with dissection of the pancreatic artery, apparently tolerating the stress of surgery poorly because of undernutrition.

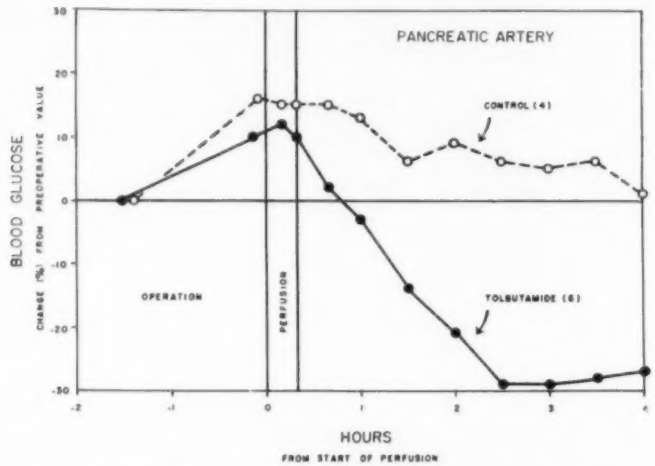

Fig. 3.—Mean curves of blood glucose following (1) pancreatic perfusion of tolbutamide in a dosage of 7 mg. per kilogram of body weight, or (2) a solution lacking sulfonylurea. The figures in parentheses refer to the number of dogs in each group.

Most of the hyperglycemia noted prior to perfusion with the different techniques was due to barbiturate anesthesia (average of all dogs, 15 per cent rise in blood glucose). There was a further increase with operation, presumably of adrenal cortical or medullary origin, which depended on the type of procedure applied. The reason for the differences in this response for the various sites is unknown. Alterations in pancreatic blood flow and disturbance of nerve pathways around the portal vein are factors which might be concerned. The fasting imposed prior to the operative procedure might have limited the capacity of the pancreas to respond to these stresses.

The question of the influence of this hyperglycemia from operation and anesthesia on the subsequent hypoglycemic response to injected agents has a

*Average increase in blood glucose following Nembutal anesthesia (prior to surgery) in all control and experimental dogs for each site of injection was as follows: pancreatic artery 20, femoral vein 13, hepatic artery 16, portal vein 5, and pancreatic artery in conjunction with duct ligation 19 per cent. These averages were calculated from the initial level of blood glucose taken before anesthesia was given.

†Average further increase in blood glucose during the operative procedure was as follows: pancreatic artery 15, femoral vein 16, hepatic artery 6, portal vein 44, and pancreatic artery in conjunction with duct ligation 31 per cent. These averages also were calculated from the initial preanesthetic level of blood glucose and represent the further increment beyond the rise due to anesthesia (see footnote above).

direct bearing on interpretation of the data. Of course, use of control animals subjected to the same stresses should help answer this objection. In addition, to determine whether anesthesia and surgery might block the peripheral effects of pancreatic insulin secretion, two animals were subjected to dissection of the pancreatic circulation under Nembutal anesthesia and were then injected

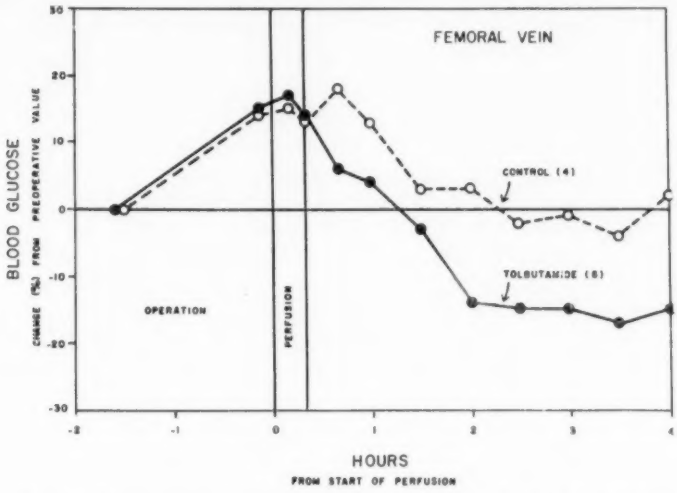

Fig. 4.—Mean curves of blood glucose following introduction of tolbutamide (6 dogs) or a control solution (4 dogs) into the femoral vein. Dosage of tolbutamide used (7 mg. per kilogram of body weight) was identical with that in the experiments depicted in Fig. 3.

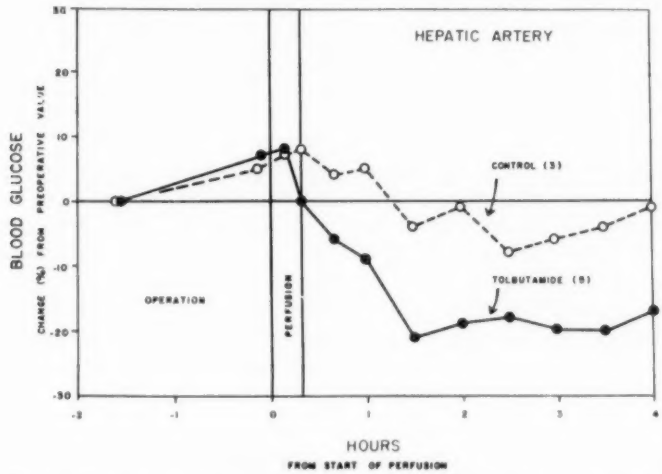

Fig. 5.—Mean curves of blood glucose after perfusion of tolbutamide (5 dogs) or a control solution (3 dogs) through the left branch of the common hepatic artery. Dosage of tolbutamide used (7 mg. per kilogram of body weight) was the same as in Figs. 3 and 4.

intravenously with 0.2 unit of crystalline insulin* per kilogram of body weight. An immediate drop in blood glucose occurred to a level of 50 per cent of the preinjection value, maximum at 40 minutes (Fig. 6). The same amount of insulin given to two unanesthetized, unoperated animals produced only a slightly greater and more sustained hypoglycemia. This indicates that a response to insulin can be demonstrated under the conditions of the surgical technique employed, despite the complicating factors mentioned above.

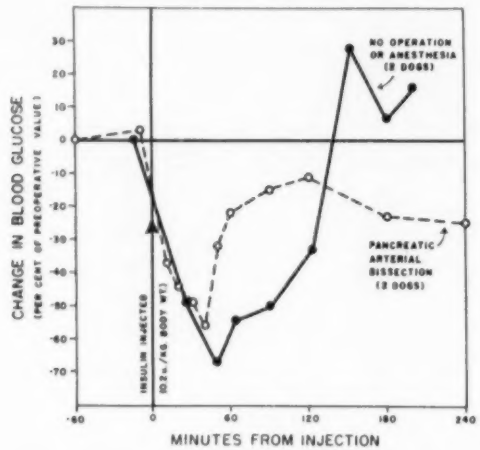

Fig. 6.—Mean fall in blood glucose from intravenous injection of crystalline insulin in a dosage of 0.2 unit per kilogram of body weight. In 2 anesthetized animals, the pancreatic artery had been isolated. Two other animals were not subjected to surgery.

Following perfusion the control (nonsulfonylurea) animals exhibited only minor differences in response depending on the site of injection utilized. Blood glucose returned to its preoperative level promptly in animals of the femoral vein and hepatic artery groups (Figs. 4 and 5), whereas it remained somewhat elevated in the pancreatic artery dogs (Fig. 3). It is possible that the arterial perfusion technique somehow reduced slightly the insulin output of the pancreas. There was no correlation in the individual animals of any group between the degree of preinjection rise and of postinjection fall in blood glucose.

A definite drop in blood glucose from the preoperative levels failed to occur with injection of the control solution into the various blood vessels. Therefore, it may be said that the hypoglycemia in the experimental animals was a result of the sulfonylurea and that the manipulative procedure did not contribute to it. Comparison of the mean reduction of blood sugar in sulfonamide-injected animals with the curves obtained on their own controls seems more valid than comparison of results of perfusion at the various sites.

Pancreatic Artery.—Pilot experiments were performed initially to establish the blood glucose response to injection of carbutamide into the pancreatic artery. Dosage ranged between 12 and 71 mg. per kilogram of body weight.

*Iletin, manufactured by Eli Lilly & Company, Indianapolis, Ind.

Table II shows that the maximum hypoglycemia produced by the drug (2 to 3 hours after perfusion) varied roughly with the dosage and the resulting blood sulfonylurea level. The amount of carbutamide was progressively reduced in order to obtain the minimum concentration which would still produce hypoglycemia, presumably by a local pancreatic effect. When a dosage of 7 mg. per kilogram of body weight was used, producing a venous blood sulfonylurea level (2 to 4 mg. per cent) too low for therapeutic effect, a fall in blood glucose occurred. If the same amount of carbutamide was injected into the femoral vein instead of the pancreatic artery, no such hypoglycemia was noted.[4] With the exception of the portal vein perfusions, all subsequent experiments utilized the same small dose (7 mg. per kilogram) of tolbutamide.

Fig. 3 illustrates the mean curves of change in venous blood glucose following perfusion of the pancreatic artery with tolbutamide or an identical solution lacking tolbutamide. Raw data for the individual experiments are given in Table III. Mean blood sugar dropped approximately 30 per cent from the preoperative value in the 6 experimental animals. The effect is somewhat greater if the control (nonsulfonylurea) curve is used for a base line of reference. Hypoglycemia failed to occur in the 4 control dogs. Both groups showed a rise in blood glucose with the stress of surgery and anesthesia. Although slightly greater in the control animals, this was generally comparable in the two groups.

Femoral Vein.—For the purpose of comparison with the results of pancreatic perfusion, 10 animals were subjected to sham operation on the arterial circulation of the pancreas and then tolbutamide or a control solution was injected into a femoral vein. Results of these studies are given individually in Table III, and mean curves are shown graphically in Fig. 4. Maximum drop in blood glucose was somewhat greater in the experimental dogs (15 per cent) than in the control group (4 per cent). However, the difference between the two curves is only half that between the experimental and control curves following pancreatic perfusion (Fig. 3). Making use of the control data, analyses showed a significant difference between the responses for the femoral vein and pancreatic artery groups.*

Hepatic Artery.—The same dose (7 mg. per kilogram of body weight) of tolbutamide (5 dogs) or a control solution (3 dogs) was perfused into the left hepatic artery. Hypoglycemia of 20 per cent occurred in the sulfonylurea-injected animals (Fig. 5), approximately the same response seen in the femoral vein group. Control dogs in which the hepatic artery was perfused with a solution lacking sulfonylurea exhibited little or no hypoglycemia. The difference between the control and experimental curves was much smaller than the response to injection of the pancreatic artery (Fig. 3). The changes following perfusion were found to be significantly different when the hepatic artery and pancreatic artery data were compared.*

*Statistical analyses utilizing mean changes from the preoperative blood glucose levels demonstrated a significant difference between values for the pancreatic artery and femoral vein groups ($p<0.01$), also between values for the pancreatic artery and hepatic artery groups ($p<0.01$). There was no difference between values for the femoral vein and hepatic artery groups.

TABLE II. RESULTS OF PERFUSION OF CARBUTAMIDE INTO THE PANCREATIC ARTERY

DOSE (MG./KG.)	MAXIMUM FALL IN BLOOD GLUCOSE (PER CENT)	MAXIMUM FREE VENOUS BLOOD SULFONAMIDE (MG. PER CENT)
71	−49	15.7
57	−36	--
16	−45	6.7
12	−28	4.1
7	−12	3.0

TABLE III. INDIVIDUAL BLOOD GLUCOSE VALUES IN DOGS BEFORE AND AFTER PERFUSION OF EXPERIMENTAL SOLUTIONS

DOG NO.	PERFUSION SITE	AGENT	BLOOD GLUCOSE (MG. PER CENT) MINUTES BEFORE OR AFTER BEGINNING OF PERFUSION											
			−90	−10	10	20	40	60	90	120	150	180	210	240
88	Pancreatic artery	Tolbutamide	88	91	92	89	92	81	61	54	44	46	47	45
89	Pancreatic artery	Tolbutamide	85	88	91	89	71	72	69	71	73	68	65	68
107	Pancreatic artery	Tolbutamide	93	112	114	112	110	106	87	72	50	59	68	76
115	Pancreatic artery	Tolbutamide	82	98	103	104	101	96	88	83	73	71	78	69
117	Pancreatic artery	Tolbutamide	88	98	94	89	79	74	76	70	72	69	66	69
118	Pancreatic artery	Tolbutamide	85	86	89	90	78	75	68	59	57	56	51	54
87	Pancreatic artery	Saline	82	96	--	90	96	97	96	98	100	93	100	80
90	Pancreatic artery	Saline	72	95	93	98	92	85	80	85	79	72	78	70
120	Pancreatic artery	Sulfadiazine	75	82	81	78	81	82	70	75	69	74	74	76
121	Pancreatic artery	Sulfadiazine	78	84	85	85	83	84	80	84	84	84	74	84
98	Femoral vein	Tolbutamide	92	111	122	121	115	112	102	84	66	62	58	58
105	Femoral vein	Tolbutamide	76	91	93	94	87	94	85	88	86	86	81	86
106	Femoral vein	Tolbutamide	80	85	88	85	83	76	72	68	67	57	61	65
114	Femoral vein	Tolbutamide	--	81	84	80	74	75	62	43	60	54	58	59
116	Femoral vein	Tolbutamide	71	83	83	79	73	65	67	61	68	69	69	68
119	Femoral vein	Tolbutamide	86	90	86	81	72	70	73	70	70	72	61	65
129	Femoral vein	Saline	73	92	98	97	102	91	80	78	72	72	69	77
130	Femoral vein	Saline	84	104	103	101	100	96	88	88	88	92	89	90
131	Femoral vein	Saline	93	96	93	91	93	96	86	90	85	84	80	89
132	Femoral vein	Saline	87	85	86	86	90	90	90	88	86	84	86	86
108	Hepatic artery	Tolbutamide	92	110	110	101	97	100	82	81	93	86	85	98
109	Hepatic artery	Tolbutamide	94	88	89	83	74	80	71	70	72	67	70	71
110	Hepatic artery	Tolbutamide	73	75	76	70	72	65	50	61	57	65	54	58
122	Hepatic artery	Tolbutamide	72	85	84	82	79	63	58	60	54	45	58	45
123	Hepatic artery	Tolbutamide	92	92	90	86	72	75	75	71	72	79	74	80
111	Hepatic artery	Saline	94	96	93	93	86	93	82	83	80	79	84	88
112	Hepatic artery	Saline	100	106	111	111	114	105	99	100	86	90	92	106
125	Hepatic artery	Saline	71	77	78	79	76	78	73	78	74	76	77	77
85	Portal vein	Carbutamide	78	125	130	133	117	139	148	136	124	116	117	107
91	Portal vein	Carbutamide	73	130	124	116	127	124	121	93	77	64	56	58
92	Portal vein	Carbutamide	96	118	144	138	134	134	128	110	102	98	94	98
86	Portal vein	Saline	86	130	150	152	154	156	156	158	128	114	102	106
133	Portal vein	Saline	85	85	85	85	86	83	85	80	80	78	71	72
136	Portal vein	Saline	78	96	88	88	79	93	78	74	78	69	78	56
137	Portal vein	Saline	83	130	130	123	111	105	89	83	75	75	79	80
124	Pancreatic artery	Tolbutamide	85	98	102	99	96	87	65	67	69	77	82	66
126	(ducts ligated)	Tolbutamide	86	114	113	110	105	103	98	77	85	86	80	81
127	Pancreatic artery	Tolbutamide	83	91	85	94	87	93	81	68	68	65	67	66
128	(ducts ligated)	Tolbutamide	92	139	138	137	128	142	123	113	96	96	94	85

Comparison of Responses.—In order to take into account the hyperglycemia from anesthesia, the experimental curves have been redrawn in Fig. 7, using the preanesthetic blood glucose as the basal reference point. It is noted that subjecting the pancreas to the sulfonylurea solution caused a definite hypoglycemia, whereas the other techniques produced little or no reduction in blood sugar below the preanesthetic level. Thus, no matter whether the response to perfusion of the experimental agents is compared to the blood glucose taken *before* or *after* anesthesia (which did cause a rise in glucose concentration averaging 15 per cent), the differences between the results of injection at the various sites are maintained.*

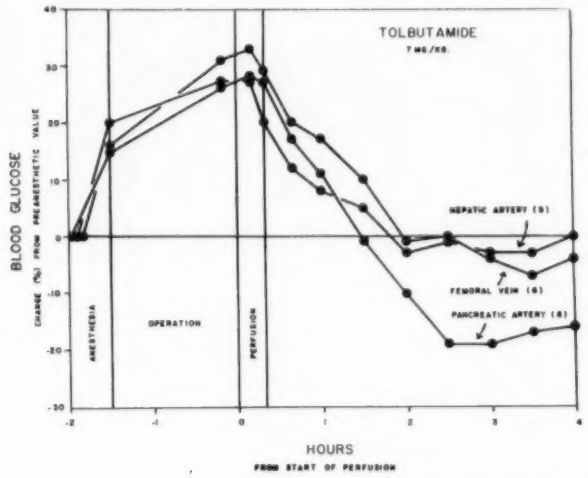

Fig. 7.—Mean change in blood glucose calculated from the preanesthetic (instead of the preoperative) value for the 3 major sites of perfusion.

Portal Vein.—Marked hyperglycemia, two or three times as great as that observed with other operative procedures, occurred when a tributary of the portal vein was cannulated (Fig. 8). Unfortunately, this effect was somewhat less in the control (saline) than in the experimental (sulfonylurea) animals, and accurate comparison is therefore impossible. However, injection of carbutamide (7 mg. per kilogram of body weight) into the portal vein failed to produce hypoglycemia.

Pancreatic Artery With Ligation of the Pancreatic Ducts.—The exeretory ducts of the pancreas were ligated in 4 animals. Six months later, the pancreatic artery was perfused with tolbutamide (7 mg. per kilogram) by the usual method. Again, preoperative rise in blood glucose was excessive (Fig. 9), but hypoglycemia was obtained. However, since control data are not available, no conclusions can be drawn.

*See footnote on page 383.

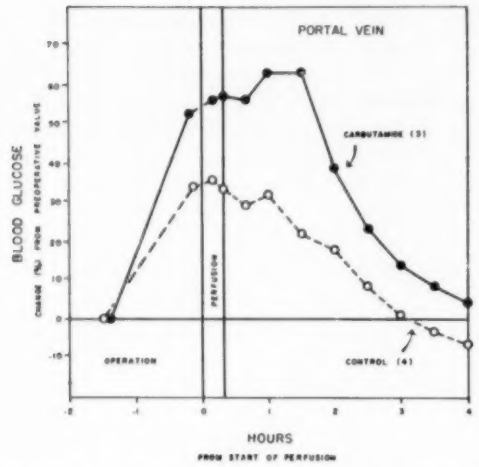

Fig. 8.—Mean curves of blood glucose after injection of carbutamide (3 dogs) or a control solution (4 dogs) into a vessel entering the portal vein. Dosage of sulfonylurea used (7 mg. per kilogram of body weight) was the same as in Figs. 3, 4, and 5.

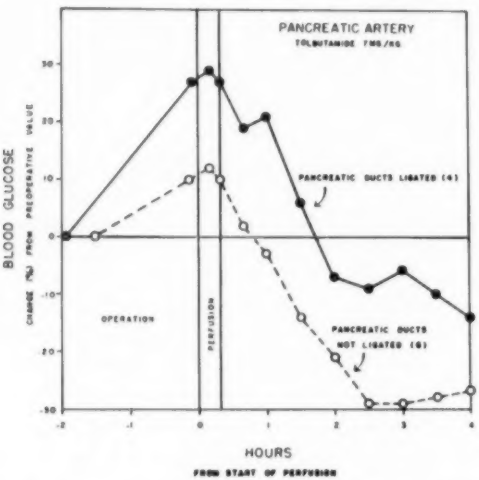

Fig. 9.—Mean curves of blood glucose after injection of tolbutamide into the pancreatic artery in animals with (4 dogs) or without (6 dogs) prior ligation of the pancreatic ducts. Dosage of tolbutamide used (7 mg. per kilogram of body weight) was the same as in Figs. 3, 4, 5, and 6.

Pancreatic Insulin Assay and Biopsies.—Results of analyses of insulin content of frozen pancreas taken from some animals are given in Table IV. Since not all specimens were assayed, these data are incomplete. Definite differences between the various experimental groups were not obtained. Pancreatic biopsies obtained from experimental and control animals injected with carbutamide failed to show alteration in morphology of alpha or beta cells.

TABLE IV. MEAN INSULIN CONTENT OF PANCREAS FOLLOWING PERFUSION OF EXPERIMENTAL AND CONTROL SOLUTIONS

NO. OF DOGS	AGENT	DOSE (MG./KG. OF B.W.)	PANCREATIC INSULIN (μ/GM.)
	Pancreatic Artery		
2	Tolbutamide	7	1.2
2	Tolbutamide	3	1.8
2	Tolbutamide	1	2.6
2	Carbutamide	7	2.7
1	Saline	--	1.9
	Femoral Vein		
3	Tolbutamide	7	2.1
2	Tolbutamide	3	2.0
4	Carbutamide	7	2.2
	Portal Vein		
3	Carbutamide	7	1.4
1	Saline	--	0.9

DISCUSSION

It was the intention of these experiments to determine the behavior of the venous blood glucose on exposure of certain organs to a low concentration of sulfonylurea. It was hoped that the site of action of these agents could thus be localized. First, moderate amounts (12 to 71 mg. per kilogram of body weight) of carbutamide were injected into the pancreatic artery. A minimal dosage (7 mg. per kilogram of body weight) was established which would produce peripheral hypoglycemia when introduced into the pancreas but not when introduced into the femoral vein. Then the same amount of tolbutamide was perfused through the pancreas or liver by way of the appropriate blood vessels. Hypoglycemic response from perfusion of the pancreatic artery was significantly greater than that from perfusion of the femoral vein or hepatic artery. This difference was more pronounced if the degree of drop in blood glucose for each site of injection was judged from its own control curve (where a solution lacking sulfonylurea was employed). No hypoglycemia was obtained from perfusion of these control solutions. The dissimilarity between the responses was borne out further when the alteration in blood glucose was calculated from the preanesthetic instead of from the postanesthetic level.

The results show that maintaining a low concentration of tolbutamide in the pancreatic circulation for a period of 20 minutes influenced the organ in some way to lower the peripheral venous blood glucose. With the dosage employed, an average of 6 mg. of tolbutamide was added to the pancreatic circulation each minute for 20 minutes. The rate of blood flow through the pancreatic

artery in the experiments is unknown. If this were postulated to be 10 ml. per minute, then a therapeutic concentration (10 mg. per cent) of sulfonylurea in the peripheral blood would supply the pancreas with approximately 1 mg. per minute. Although the sulfonylurea content of the pancreatic venous blood was not measured, it may be assumed that it was considerably greater than that of the peripheral circulation. The maximum venous free sulfonamide level in animals injected with carbutamide (7 mg. per kilogram) was only 3.0 mg. per cent (Table II), well below the effective therapeutic level. Concentrations of tolbutamide in the pancreatic and peripheral blood were undoubtedly comparable to those of carbutamide since the same dosage was administered. Injecting the same amount of the agent into the femoral vein or hepatic artery failed to produce a similar effect on the blood glucose.

Curves obtained from perfusion of the arterial circulation to the ductligated pancreas were inconclusive. Destruction of the acinar tissue did not seem to influence the usual hypoglycemic response to tolbutamide injection. The opportunity to subject similarly prepared animals to perfusion of solution without sulfonylurea was lacking. Evidence that the acini are involved in the pancreatic action of the sulfonylureas has never been presented. It is felt that the suboptimal nutritional state of the dogs, manifested by an increased hyperglycemic response to stress, may have impaired the usual functional response of the pancreas.

Results obtained from estimation of pancreatic insulin content failed to establish any consistent differences between the values for the various sites of perfusion (Table IV). However, it is interesting and suggestive that the mean values following pancreatic injection bore a rough relation to the amount of tolbutamide used. Larger doses of the drug seemed to cause a greater fall in insulin concentration. These data, although incomplete, are compatible with the idea that the sulfonylurea compounds lower the blood glucose by stimulating a release of insulin from the pancreas.

The technique of arterial perfusion described proved valuable in investigating pancreatic islet function. Test materials other than the sulfonylureas might be injected in a similar manner to assess their influence on the islet cells.

Liver Perfusion.—A marked hyperglycemia was obtained prior to perfusion with carbutamide of the portal circulation via one of its tributaries. There was no sign of a peripheral hypoglycemia from exposing the liver to the carbutamide. Moreover, perfusion of tolbutamide (7 mg. per kilogram) into the left hepatic artery caused little or no fall in blood glucose from the preoperative level (Fig. 5). These results constitute strong evidence against an hepatic action as the primary reason for the peripheral hypoglycemia caused by the sulfonylureas. They also indicate that the drugs do not act on the liver to cause hypoglycemia after leaving the pancreas. The liver may have a role in the blood glucose alteration from these agents, but this function is probably secondary to insulin release by the pancreas.

REVIEW OF THE LITERATURE

A critical evaluation of the evidence in the literature regarding clinical and animal experiments with the sulfonylureas bears out the hypothesis of a pancreatic site of action. The presence of endogenous insulin is necessary for the hypoglycemic effect. Influences on other organs, notably the liver and thyroid gland, are also seen, particularly after high doses. Hepatic effects are complicated and may be dependent on pancreatic function. No organ except the pancreas is essential for the hypoglycemic response. The agents have no influence on blood glucose in the eviscerated animal.[10] It is our intention to examine briefly the important data which support these general statements. Table V summarizes salient findings by workers interested in this problem.

Adrenal.—Neither the adrenal cortex nor the adrenal medulla needs to be available for the sulfonylureas to be effective.[11-13] Total adrenalectomy potentiated the fall in blood glucose following the administration of the compounds; this was not true after isolated removal of either cortex or medulla. Steroid excretion in the urine was not altered by carbutamide or tolbutamide therapy in human beings.[14-16]

Pituitary.—Following hypophysectomy, there was a normal response to sulfonylurea administration.[11, 12, 17]

Thyroid.—Although depression of thyroid activity as measured by the radioactive iodine uptake and blood protein-bound iodine level has been described after large doses,[18] ordinary therapeutic amounts had no such effect.[9]

Liver.—Presence of the liver is not essential to obtain a blood glucose response from the sulfonylureas.[20] Glucose output from the liver is reduced,[21-23] but attempts to link this phenomenon with inhibition of specific enzyme systems have been fruitless. The glycolytic enzymes phosphorylase and glucose-6-phosphatase have been studied extensively, and the consensus is that neither is significantly affected with the doses commonly used.[24-26] Glucagon and epinephrine hyperglycemia, presumably mediated through the phosphorylase system, is not altered by the sulfonylureas.[27, 28] Changes in liver glycogen are variable depending on the experimental procedure and dosage used.[9, 29, 30] Mirsky's early observations that "insulinase" is inhibited[31] have been contradicted by others employing smaller amounts of the drugs.[32, 33] The rate of degradation of insulin tagged with I^{131} is not altered.[32] Although the sulfonamides may antagonize certain inhibitors of insulin, there is no proof that this is related to their hypoglycemic effect.

The major action of the sulfonylureas on the liver appears to be the depression of glucose output. Whether this is primary or secondary to insulin stimulation has not been determined. High concentrations of the drugs may produce nonspecific enzyme inhibitory effects, some of which may be responsible for reducing hepatic glucose manufacture. None of these has been demonstrated to prevail at reasonable dosages or to account for the fall in peripheral blood glucose.

Insulin elaborated through the pancreatic vein into the portal system could well account for the decrease in hepatic glucose output following administration of sulfonylurea. The degree of participation of the liver in the hypoglycemic response to insulin is not well understood.[4] Frawley and associates[35] feel that the sulfonylurea compounds may potentiate the action of insulin on the liver.

Massive doses of carbutamide and tolbutamide have a toxic action on the liver which is exaggerated by removal of the pancreas. Fatty liver, hepatic

TABLE V. CRITICAL EVIDENCE REGARDING SITE OF ACTION OF THE SULFONYLUREAS

PREPARATION	FINDING		REFERENCES
	Adrenals		
Man	No influence on urinary steroid excretion or blood electrolytes		14, 15, 16
Rat	Increased hypoglycemic effect after adrenalectomy		11, 12, 13
	Pituitary		
Dog, cat	Usual hypoglycemic response after hypophysectomy		11, 12, 17
	Thyroid		
Man	No change in RAI uptake, PBI, or BMR with usual doses		18, 19
	Liver		
Dog, man	Decrease in glucose output		21, 22, 23
Dog	Normal hypoglycemic response after hepatectomy		20
Rabbit	Increase in liver glycogen		30
Dog, rat	Decrease in liver glycogen		9, 12, 13
Rabbit liver	No reduction in glucose-6-phosphatase or phosphorylase in physiologic doses		24, 25, 26
Rat liver	No inhibition of insulinase production		32, 33, 60
	Pancreas—(Alpha Cell)		
Rat	No effect on alloxan diabetes		13, 17, 39
Man, rabbit	No change in morphology		28, 40
Rabbit, man	No influence on glucagon hyperglycemia		24, 27, 28
	Pancreas—(Beta Cell)		
Dog, cat	No effect in depancreatized animals		11, 17
Man	Effective in partially depancreatized patients		38
Man	Response varies with pancreatic insulin content		41, 42
Dog	Reduction in pancreatic insulin content		9
Rat	Increase in islet volume and weight		43
Rabbit, rat	Degranulation of beta cells		61
Dog	Hypoglycemic response in recipient animal in cross-circulation experiment		44
Rat	Rise in plasma insulin		45
Dog	Increase in pancreatic venous insulin		46
	Insulin Effects	*Positive*	*Negative*
Man	Hypoglycemia	2, 27, 52	
Man, dog	Decrease in potassium and phosphorus of blood	47, 48	
Man	Increased arteriovenous glucose difference	48, 50	22
Rabbit, rat	Change in muscle and liver glycogen	30, 54	9
Man	Change in blood pyruvate	23	53
Rat	Increase in glucose oxidation	54	49
Man	Change in glucose tolerance curve		27
Man	Increase in respiratory quotient		52, 55
Dog, rabbit	Increase in intracellular transfer of glucose or galactose		49
Rat	Increase in glucose uptake by muscle and fat		56, 57
Man	Correction of negative nitrogen balance		14, 55

necrosis, hypoprothrombinemia, and hemorrhage have been described in depancreatized dogs.[36, 37] This may be related to the nutritional deficiencies that occur when there is loss of the digestive functions of the pancreas.

Pancreas.—The sulfonylureas are ineffective in totally depancreatized animals.[11, 17] Hypoglycemic response to the compounds is normal in partially pancreatectomized man.[38] That the alpha cells of the islets or their function of elaborating glucagon are not involved is shown by the lack of effect in alloxan diabetes,[17, 39] the absence of any morphologic damage to the alpha cells after oral administration,[28, 40] and the failure of the sulfonamides to influence the hyperglycemic response to injected glucagon.[27, 28]

Functioning beta cells must be available for a response to occur. There is a high correlation between the amount of insulin extractable from the human pancreas and the occurrence of a decline in blood glucose as a result of administration of the sulfonylureas.[41] Patients with juvenile diabetes, known to exhibit a low pancreatic insulin content, are largely unresponsive except early in the course of their disease.[42] Ashworth and Haist's[43] observation that an increase in islet volume and weight occurs after sulfonylurea administration is consistent with the hypothesis of a stimulus to insulin secretion. Experiments on cross circulation also support this concept.[44] Degranulation of beta cells has been observed after prolonged treatment,[28] but whether this represents insulin stimulation or functional rest is difficult to decide. Toxic doses cause a reduction in pancreatic insulin content in the experimental animal, but therapeutic doses do not.[9] Von Holt and associates[45] have demonstrated a rise in plasma insulin in acute experiments on rats. Goetz and Egdahl[46] recently found that tolbutamide produced an increase in insulin output into the pancreatic vein. To date, there is no convincing evidence that these compounds cause an "exhaustion" of pancreatic islet cells by means of repeated stimulation of insulin release.

Despite the evidence cited favoring an action of the sulfonylureas involving insulin synthesis or release, the usual metabolic effects of injected insulin are strangely lacking. That the normal patient develops a striking hypoglycemia and the diabetic a lesser response is agreed. Two well-controlled studies show a decrease in blood potassium and phosphorus similar to that following insulin.[47, 48] The depression of phosphorus after insulin injection implies entry of glucose in the cell; yet, Wick and co-workers[49] were unable to show in eviscerated animals that the sulfonylureas cause an increased intracellular transfer of glucose. There are, also, other contradictory data. Goetz and associates[48] and Madison and Unger[50] found that the sulfonylureas cause an increased arteriovenous glucose difference, again implying cellular entry of glucose. Purnell and co-workers,[22] with equally well-controlled experiments, were unable to demonstrate such an effect. Muscle and liver glycogen data are particularly confusing, with results reported which are diametrically opposed.[9, 12, 13, 30, 51] Undoubtedly variations in technique play a large role in the glycogen evidence. Dulin and Johnston[20] found that they could produce hypoglycemia by means of small intravenous doses of insulin without alteration in liver glycogen, an effect

comparable to that of tolbutamide. Tolbutamide was found to lower blood ketones and plasma unesterified fatty acids in diabetic patients.[52] However, evidence regarding fat metabolism is largely inadequate. Hennes and associates[53] described a fall in blood pyruvate immediately following tolbutamide administration, in contrast to the early rise after insulin. Using insulin doses calculated to produce the same hypoglycemic effect as the sulfonylureas, Miller and his co-workers[23] were unable to demonstrate any difference in pyruvate response. If the agents stimulate insulin release, one might expect an increased oxidation of glucose by the peripheral tissues. Miller and associates[54] were able to show this by carbon labeling, but Wick and associates[49] were unable to do so. Insulin effects which could *not* be demonstrated employing the sulfonylureas include correction of negative nitrogen balance,[14] increase of respiratory quotient,[55] improvement in glucose tolerance,[27] and increased glucose uptake by muscle[26, 29, 56] and adipose tissue.[57] Certainly, the compounds are not truly antidiabetic since they fail to correct the metabolic abnormalities resulting from insulin deficiency. Indeed, this fact mitigates against our hypothesis that they cause hypoglycemia by releasing pancreatic insulin.

The data presented in this article, as well as the evidence cited, favor a primary influence of the sulfonylurea compounds on the pancreas. Introducing a very small amount of tolbutamide (average, 6 mg. per minute for 20 minutes) into the pancreas provoked a hypoglycemic effect, whereas injection of the same dose into the liver or a peripheral vein did not. Urgoiti in Houssay's laboratory injected larger dosages of tolbutamide into the pancreatic artery by a different technique and was unable to demonstrate definitely a stimulating effect.[58] However, a greater drop in blood glucose was found with pancreatic artery injection than with saphenous or portal vein injection (not statistically significant). Loubatières[59] produced hypoglycemia by injection of the isopropylthiodiazole derivative of sulfanilamide (40 mg. per kilogram of body weight) into the pancreatic artery or pancreatic duct.

It is our view that tolbutamide or carbutamide in the pancreatic arterial blood cause insulin to be released in a slow, sustained fashion from the beta cells of the islets into the pancreatic vein so that there is a reduction in hepatic and therefore peripheral venous blood glucose. Some of the contradictions in the experiments mentioned might be explained by the timing, dosages, and species differences involved. It may be impossible to demonstrate the peripheral effects of insulin on tissue or muscle by present techniques if small amounts are released slowly by the pancreas.

SUMMARY

Site of action of the sulfonylureas was investigated by injecting solutions of the compounds into the blood vessels supplying the pancreas and liver. Perfusion of the pancreas via its arterial circulation with a small amount of tolbutamide in the anesthetized dog caused moderate hypoglycemia. Little or no such effect was obtained when the femoral vein or hepatic artery was perfused with the same amount (7 mg. per kilogram of body weight) of the drug. Mean response of the pancreatic artery group was significantly different from

those of the femoral vein or hepatic artery groups. The primary importance of the liver in the response was further refuted by a lack of hypoglycemia following injection of carbutamide into the portal vein. Although ligation of the pancreatic ducts failed to prevent the hypoglycemic response to sulfonylurea introduced into the pancreatic circulation, no conclusions may be drawn regarding an acinar role because of absence of control information. There was no alteration in morphology of alpha or beta cells following perfusion of the main pancreatic artery with carbutamide.

The important literature bearing on the question of the mechanism of action of these compounds is reviewed, and the nature of their influence on the pancreas is discussed. The data support the idea that the sulfonylurea compounds cause a decrease in blood glucose by virtue of their effect on the pancreatic islets. These experiments tend to establish the usefulness of the technique of pancreatic perfusion for estimating the effect of various substances on islet function.

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THE EFFECT OF PREDNISONE AND AMPHENONE ON FLUID AND ELECTROLYTE BALANCE AND ON ALDOSTERONE EXCRETION OF PATIENTS WITH CIRRHOSIS AND ASCITES

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THE accumulation of ascites and edema by patients with chronic liver disease is associated with a low sodium content of urine, sweat, and feces,[1-4] and adrenal hormones have been held responsible in part for this phenomenon. Recent observations have given added support to this hypothesis. Aldosterone, a potent salt-retaining hormone isolated from adrenal venous blood,[5] is excreted in excessive amounts by patients with cirrhosis and ascites.[6-8] Moreover, bilateral adrenalectomy has been performed for treatment of rapidly accumulating ascites in human beings with cirrhosis,[9, 10] although this seems a formidable procedure when rigid sodium restriction alone will halt further ascites formation.[1, 2, 11]

Amphenone†[12, 13] inhibits adrenal cortical steroid secretion in animals and man.[14-18] The clinical usefulness of this compound is hampered by its toxic effects (i.e., skin rash, drowsiness, gastrointestinal symptoms, methemoglobinemia, and impaired liver function tests). However, Renold and associates[19] have found only mild toxic manifestations in man when Amphenone was given for only a short time.

To help elucidate the role of adrenal cortical steroids in the pathogenesis of sodium retention as observed in cirrhosis with ascites, Amphenone was given to inhibit steroid production. The untoward side effects of this drug and the potential hazard of adrenal insufficiency necessitated the coadministration of prednisone which provided the opportunity to evaluate its diuretic properties. Thus, prednisone and Amphenone were administered to a selected group of patients resistant to standard diuretic therapy. The effect of these drugs on fluid balance and on urine sodium and aldosterone excretion was studied.

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MATERIALS AND METHODS

Ten patients with documented cirrhosis complicated by ascites and edema were selected for study. The group comprised of 7 men and 3 women, aged 40 to 67 years, who were resistant to diuretic therapy with salt restriction and mercurials. Diagnosis was established by history, physical examination, and laboratory studies in each case. In 7 instances, confirmatory histologic evidence was also available. Nine patients gave a history of chronic alcoholism and poor diet for from 6 to 40 years, and the remaining patient (Case 7) had cirrhosis of undetermined etiology confirmed by liver biopsy. A history of previous jaundice, which seemed related to acute hepatic decompensation of the alcoholic, was elicited in 2 patients. Ascites had been present continuously for from 2 to 18 months prior to study, although 7 patients had had persistent ascites of only 3 months' duration or less. Three patients had previously had paracenteses and 3 gave a history of intermittent ascites over a 1- to 6-year period. All patients showed evidence of poor nutrition; severe wasting was present in 6, while 2 were obese. Ascites was severe in all cases. Case 10 showed signs of mild cardiorespiratory insufficiency which was relieved as ascites improved. Accurate estimation of liver and spleen size was prevented by ascites. X-ray examination of the esophagus did not reveal varices in any patient; however, 7 had enlarged abdominal collateral veins and 4 had hemorrhoids. A history of recent impending hepatic coma was present in 3 cases (Cases 4, 8, and 10), and Case 1 had arteriosclerotic heart disease with atrial fibrillation, which was well stabilized with maintenance digitalis throughout the study.

All patients were ambulatory on the Thorndike metabolic ward and were maintained on a constant diet supplying 10 mEq. or less of sodium, approximately 100 mEq. of potassium, 50 to 80 Gm. of protein, and at least 2,200 calories daily as calculated from standard tables.[20] Direct analysis of a daily diet was performed on 2 occasions revealing 4.6 and 4.9 mEq. of sodium, 109 and 100 mEq. of potassium, and 9.5 and 11.3 Gm. of nitrogen for Cases 9 and 10, respectively. Fluids were allowed ad libitum, except for Cases 5 and 6 who were restricted to 1,600 ml. and 2,000 ml. per day, respectively. The studies were made during cool weather in all but 3 patients (Cases 1, 2, and 3), minimizing variations in skin losses of sodium and water. Fecal and sweat losses of sodium were assumed to be 8 to 10 mEq. per day[11] and the remaining insensible fluid loss to be 800 to 1,000 ml. per day. No patient experienced diarrhea or vomiting during the study.

The patients were weighed at the same time each day. Daily fluid intake was recorded. Total 24-hour urine output was measured and daily aliquots taken for determination of sodium, potassium, chloride, and nitrogen. In selected cases samples were saved for aldosterone determination, and a sample was frozen for osmolality measurements. Blood specimens were obtained from an antecubital or femoral vein without stasis. Determinations of hematocrit, serum potassium, sodium, chloride, albumin, nonprotein nitrogen, and bilirubin were made before each change in therapy and at other indicated times. Daily clinical evaluations were made and recorded.

Methods used in this laboratory for the above determinations have been previously recorded,[21, 22] with the exception of serum albumin, which was measured by the method of Rutstein and associates.[23] Total solute concentration in urine and serum was measured by freezing-point depression using a Fiske osmometer, and results are recorded as osmolal concentrations.

Urinary aldosterone excretion was measured by a modification[24] of the Neher and Wettstein method.[25] Unconjugated aldosterone is known to be easily destroyed when exposed to strongly acid conditions.[26] Thus, in view of the possibility that impaired liver function might result in defective conjugation of aldosterone, all but 3 urine specimens were extracted twice: (1) within one hour after acidification to pH 1 (this fraction is designated "free" aldosterone), and (2) again 48 hours later (this fraction is designated "conjugated" aldosterone). The combined extraction, performed on 6 urines of patients

TABLE I. METABOLIC BALANCE STUDIES IN 10 PATIENTS WITH CIRRHOSIS AND ASCITES

PATIENT, AGE, SEX, WEIGHT AND DATE	DAY OF STUDY	PERIOD OF STUDY*†	MEAN WEIGHT CHANGE PER DAY (KG.)	MEAN 24 HR. URINE					MEAN 24 HR. FLUID INTAKE (ML.)
				SODIUM (MEQ.)	POTASSIUM (MEQ.)	CHLORIDE (MEQ.)	NITROGEN (GM.)	VOLUME (ML.)	
1. 62, M 79.6 Kg. 8/18/56	1-6	I	−0.1	2	58	28	---	1,175	---
	7-9	II	−0.06	5	74	24	---	1,536	---
	10-12	III	+0.2	13	58	51	---	944	---
	13-15	IV	−0.46	49	116	101	---	1,830	---
	16-17	V	+0.3	2	30	35	---	1,180	---
2. 40, M 105 Kg. 8/11/56	1-6	I	−0.26	5	28	24	---	896	---
	7-9	II	−0.33	1	50	7	---	1,326	---
	10-12	III	−0.33	1	69	14	---	2,298	---
	13-16	IV	−0.30	1	78	19	---	2,402	---
	17-18	V	−0.55	44	101	8	---	1,670	---
	26-32	IIa	−0.2	1	68	24	---	1,981	---
3. 67, M 72 Kg. 9/6/56	1-6	I	+0.28	1	30	6	---	830	2,116
	7	A	−0.5	1	32	2	---	390	2,135
	8-10	II	−0.9	7	77	16	---	1,360	1,758
	11-14	III	−0.25	25	84	37	---	991	2,000
	15-18	IV	−0.5	12	48	34	---	681	1,880
	19-25	V	+0.29	10	36	--	---	587	1,670
	26-30	IIa	−0.18	1	23	3	---	765	1,877
	31-35	Va	−0.1	1	36	2	---	617	1,851
4. 42, F 47 Kg. 10/11/56	1-7	I	+0.01	1	18	3	---	418	1,444
	8-15	II	+0.13	1	24	5	6.1	899	1,686
	16-21	V	−0.12	55	54	30	4.8	962	1,949
	22-25	Vb	+0.17	1	30	2	5.0	735	1,615
5. 46, M 62 Kg. 10/31/56	1-7	I	−0.05	1	20	4	3.4	516	1,648
	8-16	II	−0.41	33	35	33	4.8	971	1,596
	17-22	V	−0.29	45	36	38	3.1	840	1,600
6. 42, F 64 Kg. 1/14/57	1-5	I	−0.00	12	63	16	3.8	1,445	1,985
	6-17	II	−0.42	61	71	67	5.4	2,069	2,091
	18-26	V	−0.23	44	57	52	---	2,141	2,000
7. 63, F 84 Kg. 1/26/57	1-7	I	+0.26	2	39	10	4.6	1,115	2,214
	8-17	II	−0.02	14	40	19	4.5	1,566	2,704
	18-20	III	−0.17	35	41	50	5.1	1,540	2,813
	21-26	IV	+0.05	18	44	47	6.4	1,512	2,650
	27-37	IVc	+0.06	3	43	12	5.3	1,400	2,644
	38-42	V	+0.16	1	38	38	---	966	2,640
8. 42, M 63 Kg. 2/7/57	1-6	I	+0.03	7	28	45	---	988	2,080
	7-14	II	−0.01	1	33	18	---	1,284	2,105
	15-18	III	−0.25	29	56	38	---	1,490	2,485
	19-20	IV	−0.7	36	61	47	---	1,020	1,870
9. 40, M 81 Kg. 3/1/57	1-6	I	+0.26	2	54	24	4.9	783	1,555
	7-17	II	−0.64	68	111	101	7.7	1,966	1,719
	18-23	III	−0.81	67	114	129	7.0	2,001	2,001
	24-30	IV	+0.65	4	96	26	8.9	1,346	1,825
	31-36	V	+0.07	1	89	22	8.7	859	1,441
10. 42, M 82 Kg. 3/13/57	1-10	I	+0.02	2	43	21	5.6	323	2,323
	11-17	II	−0.00	1	84	6	7.7	947	2,212
	18-24	III	−0.75	105	92	99	9.5	1,712	2,046
	25-31	IV	−0.2	15	102	16	8.5	744	1,938
	32-34	V	+0.5	1	92	2	7.5	530	2,218

Note: The data recorded for each period represent the average of serial 24-hour determinations.

*Period I = control (mercurial diuretics were given); Period II = prednisone administration; Period III = prednisone and Aminophylline administration; Period IV = prednisone administration; Period V = final control (mercurial diuretics were given); Period A = Aminophylline administration. See text for dosage.

†a = repeat study; b = no mercurial diuretic given; c = addition of mercurial diuretics every third day; in the other studies mercurials were given during Periods I and V only.

without cirrhosis, revealed a mean ratio for "free" to total ("free" plus "conjugated") of 20 per cent ranging from 1 to 40 per cent (Table III). To check for purity of measured aldosterone, several aliquots of the samples containing aldosterone at the end of the procedure[24] were chromatographed on the "E_2B" system of Eberlein and Bongiovanni.[27] From this it appeared that most of the samples examined did not contain any of the contaminants found by Nowaczynski and associates[28] (labeled p in Table III). The small amounts of contaminating material found in the few remaining cases (labeled c in Table III) did not interfere with the final quantitative reaction used for aldosterone, as they did not immediately reduce blue tetrazolium.

Prednisone was given before, during, and after Amphenone. All patients received 30 mg. per day, with the exception of Cases 1 and 2 who received 50 mg. per day. Seven patients received Amphenone while 3 others received prednisone alone. The average daily dose of Amphenone was as follows: Case 8, 3.1 Gm.; Case 9, 3.6 Gm.; Case 3, 4.5 Gm.; Case 2, 5 Gm.; Case 1, 5.6 Gm.; Case 10, 5.6 Gm.; and Case 7, 6.0 Gm.

The study was divided into 5 periods: Period I, control (no medication except mercurials), 5 to 10 days; Period II, prednisone, 3 to 12 days; Period III, prednisone and Amphenone, 3 to 7 days; Period IV, final prednisone, 3 to 7 days; and Period V, final control (no medication except mercurials), 2 to 7 days. Mercaptomerin sodium was given once or twice to all patients except Case 9 during Period I and again during Period V. Case 8 left the hospital against advice while receiving prednisone without a final control period. Four patients received a second course of prednisone at a later date. Some of the data on Cases 1, 2, 3, 7, 8, and 10 are included in previous reports from this laboratory.[22, 29]

RESULTS

Metabolic Balance Studies.—Daily results have been averaged for each period and are shown in Table I. The balances are approximate, as stool and insensible losses were not measured. (See Materials and Methods.)

A. Period I, control (no medication except mercurial diuretics): Only slight changes in weight, sodium, potassium, or water balance were found during this period. Weight loss was small and considered insignificant. The greatest weight loss, an average of 0.22 Kg. per day in Case 2, resulted from a 2 Kg. loss in response to mercurial injection. Small weight gains were noted in 3 patients. Mean daily urine sodium was 3.9 mEq. per day, and excluding mercurial responses was 1.9 mEq. per day. Average urine volumes were less than 1,000 ml. per day for 7 of 10 patients, and between 1,000 and 1,500 ml. for the remaining 3.

B. Period II, prednisone: During this period a weight loss greater than 0.25 Kg. per day occurred in 5 of 10 patients (Cases 2, 3, 5, 6, and 9), varying from 1 Kg. in 3 days (Case 2) to 7.1 Kg. in 11 days (Case 9). All patients showed an increase in average urine volume of 350 ml. per day or more (Fig. 1). A decrease of estimated water balance greater than 350 ml. per day occurred in 6 of 8 patients (Cases 3, 5, 6, 8, 9, and 10).

Fluid intake data were incomplete for Cases 1 and 2. A moderate increase of fluid intake occurred only in Cases 4 and 7 (Table I), although only Cases 5 and 6 were restricted in fluid intake. Urine volume bore no relation to urine sodium concentration, except that the 2 patients with volume

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1. 62, M	1-6	I	−0.1	2	58	28	---	1,173	---
79.6 Kg.	7-9	II	−0.06	5	74	24	---	1,536	---
8/18/56	10-12	III	+0.2	13	58	51	---	944	---
	13-15	IV	−0.46	49	116	101	---	1,830	---
	16-17	V	+0.3	2	30	35	---	1,180	---
2. 40, M	1-6	I	−0.26	5	28	24	---	896	---
105 Kg.	7-9	II	−0.33	1	50	7	---	1,326	---
8/11/56	10-12	III	−0.33	1	69	14	---	2,298	---
	13-16	IV	−0.30	1	78	19	---	2,402	---
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B. Period II, prednisone: During this period a weight loss greater than 0.25 Kg. per day occurred in 5 of 10 patients (Cases 2, 3, 5, 6, and 9), varying from 1 Kg. in 3 days (Case 2) to 7.1 Kg. in 11 days (Case 9). All patients showed an increase in average urine volume of 350 ml. per day or more (Fig. 1). A decrease of estimated water balance greater than 350 ml. per day occurred in 6 of 8 patients (Cases 3, 5, 6, 8, 9, and 10).

Fluid intake data were incomplete for Cases 1 and 2. A moderate increase of fluid intake occurred only in Cases 4 and 7 (Table I), although only Cases 5 and 6 were restricted in fluid intake. Urine volume bore no relation to urine sodium concentration, except that the 2 patients with volume

increases of over 600 ml. per day (Cases 6 and 9) also excreted the greatest amount of sodium.

A brisk sodium diuresis occurred in 3 of 10 patients (Case 5, 33 mEq. per day; Case 6, 61 mEq. per day; Case 9, 68 mEq. per day), and 3 others showed small but definite increases of sodium excretion (Case 1, 5 mEq. per day;

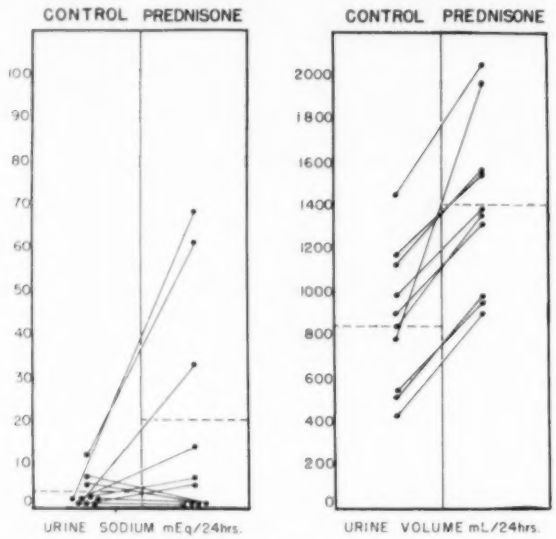

Fig. 1.—Changes in urine volume and urine sodium excretion of 10 patients with cirrhosis and ascites during prednisone administration.

Case 3, 7 mEq. per day; Case 7, 14 mEq. per day). The other 4 patients continued to excrete less than one mEq. per day (Fig. 1). Case 2 was the only patient to lose weight without increasing urine sodium. Sodium and water diuresis began from 1 to 4 days after initiation of prednisone, with the maximum effect between the fourth and tenth days. Patient 9 exhibited the most marked diuresis in response to prednisone (Fig. 3). It should be noted that Case 3, the first patient studied, received Amphenone alone on the day prior to the administration of prednisone, and during a later course of prednisone alone he failed to show a natriuresis, so that the effect observed during Period II may have been related to Amphenone administration. An increase in urine sodium can thus be attributed to prednisone in 5 of 10 patients, with a clinically significant loss in 3.

Increases in potassium excretion greater than 25 mEq. per day occurred only in Cases 9 and 10. Correction of potassium excretion using the changes in urine nitrogen* showed that the increased urine potassium in these 2

*The figures for daily urine potassium excretion were corrected for prednisone-induced cellular catabolism in the following manner. The increase in urine nitrogen (in grams) during the prednisone period multiplied by 2.7 was assumed to be equal to potassium (expressed as milliequivalents) from cellular breakdown.

patients was more than could be accounted for by increased cellular catabolism. Average urine solute concentration rose in some instances and fell in others with no relation to urine volume (Table II).

TABLE II. MEAN URINE AND SERUM SOLUTES FOLLOWING PREDNISONE ADMINISTRATION

PATIENT	PERIOD OF STUDY	URINE			SERUM	
		VOLUME (ML./24 HR.)	SODIUM (MEQ./24 HR.)	SOLUTES (MOSM./L.)	SODIUM (MEQ./L.)	SOLUTES (MOSM./L.)
5	I	516	1	243	127	273
	II	970	33	326	138	284
6	I	1,443	12	255	142	277
	II	2,069	61	239	140	290
9	I	783	2	---	135	288
	II	1,966	68	---	139	296
7	I	1,115	2	245	135	284
	II	1,566	14	252	136	291
8	I	988	7	---	132	274
	II	1,384	1	---	138	288
10	I	523	2	492	131	285
	II	947	1	480	134	294

Note: The urine data recorded for each period represent an average of serial 24-hour determinations, while the serum data represent the last determination during the period.

C. Period III, Amphenone and prednisone: During Amphenone administration 4 of 7 patients showed weight loss of from 0.17 to 0.75 Kg. per day and an increase in urine sodium excretion of from 18 to 104 mEq. per day. Case 2 continued to excrete less than 1 mEq. of sodium per day while losing weight, and Case 1 increased sodium excretion slightly (to 13 mEq. per day) while gaining weight. Although a marked natriuresis occurred in Case 9 during prednisone administration, the urine sodium excretion reached its maximum on the first day of Amphenone administration and then fell off in spite of continued therapy. By the end of the Amphenone period, he was clinically free of ascites (Fig. 3). Fluid balance became significantly more negative only in Case 10, who was the only patient to receive Amphenone in full 6 Gm. doses for more than 3 days. Average urine potassium excretion increased from 1 to 25 mEq. per day in all patients but Case 1.

Period II was continued for at least 8 days for the majority of patients, permitting evaluation of the effect of prednisone administration. However, the possibility that the 3-day course of prednisone used in Cases 1, 2, and 3 could have initiated a diuresis made evaluation of a subsequent Amphenone effect difficult, as a diuresis began on the third or fourth day of prednisone administration in some patients. To define the extent of the prednisone effect, these 3 patients were each given a second course of prednisone alone 3 weeks to 6 months later. After 5 to 8 days of prednisone, Case 3 (in 2 separate trials) showed no response in sodium or fluid balance, while Case 2 (in one trial) again showed an increase in urine volume and weight loss without an increase in sodium excretion. Case 1 showed a decrease in ascites and weight loss; however, urine collections were incomplete.

Thus, an unequivocal natriuresis in response to Amphenone occurred in 4 of 7 patients (Cases 3, 7, 8, and 10). Interpretation of the response in 2

TABLE III. URINE ALDOSTERONE EXCRETION

PATIENT	PERIOD	DAY	URINE ALDOSTERONE* (μG/24 HR.) FREE	CONJUGATED	TOTAL	PER CENT FREE	% OF PERIOD I
A. Patients With Cirrhosis							
9	I	Mean	5[c]	10	15	33	—
	II	15	9	10	19		
		16	4.5	12[p]	16		
		17	0	7.5	7.5		
		Mean	4.5	9.8	14	32	95
	III	18	—	—	6		
		19	—	—	7		
		20	—	—	4.5		
		Mean	—	—	5.8	39	39
	III	23	12[p,c]	20[p,c]	32[c]	37	213
	IV	24	28	25[p]	53		
		25	10	28[p]	38		
		26	15	22[p]	37		
		Mean	18	25	43	42	283
	V	32	5	40[p]	45		
		34	30	16[p]	46		
		Mean	17	28	45	38	303
10	I	Mean 1 to 10	18[c]	25[p]	43	42	—
	II	Mean 11 to 17	15[c]	28[p]	43	35	100
	III	Mean 18 to 24	2	2	4	50	9
	IV	Mean 25 to 31	0	4	4	—	9
	V	Mean 32 to 34	26[p]	23[p]	49	53	114
1	I	2	35	20	55		
		3	5.5	2.5	8		
		5	17	2	19		
		Mean	19	8	27	70	—
	II	7	5	26	31		
		8	8	0	8		
		9	35	4.5	39		
		Mean	16	9.8	26	61	96
	III	10	0.5	2.5	3		
		11	2.0	0	2		
		12	2.5	1.5	4		
		Mean	1.6	1.3	3	53	11
	IV	13	6.5	—	—		
		14	4.0	—	—		
		Mean	5.2	—	—		
2	I	1	36	14	50		
		3	25	22	47		
		6	21	18	39		
		Mean	27	18	45	60	—
	II	8	59	10	69		
		9	30	—	—		
		10	15	2	17		
		Mean	35	6	43	81	95
	III	11	2.5	4	6.5		
		12	4.0	5	9.0		
		13	0.5	5	5.5		
		Mean	2.3	4.6	7.0	33	15
	IV	14	5	25	30		
		15	20	20	40		
		Mean	12.5	22.5	35	36	77

TABLE III—CONT'D

DIAGNOSIS AND NUMBER OF PATIENTS	URINE ALDOSTERONE (μG/24 HR.)			PER CENT FREE
	FREE	CONJUGATED	TOTAL	
B. Patients Without Liver Disease				
Edema of unknown origin (1)	0.5	65	65.5	1
Congestive cardiac failure (1)	2	6	8	25
Pregnancy (1)	0.5	6	6.5	8
Unclassified edema (1)	1	3	4	25
Normal men after 1 week of sodium restriction (pool)	5†	7†	12†	41
Patients with Addison's disease given intravenous aldosterone (pool)	2†	8†	10†	20

Note: all figures represent determinations performed on 24-hour urine samples except for Subject 10 (Periods I to V), Subject 9 (Period I), and 2 pools of patients without liver disease where the determinations were performed on pooled daily aliquots.

*Key to symbols: c = sample found to be contaminated after additional chromatography on "E_2B" paper system; p = sample found to be pure after additional chromatography on "E_2B" paper system; + = figures not used in calculating mean values since Amphenone dosage was reduced to 1.5 Gm. per day during Days 21 to 23.

†Data expressed as micrograms of aldosterone per liter.

(Cases 1 and 9) was complicated by a concomitant prednisone effect, and no natriuresis occurred in another (Case 2). Only one patient (Case 10) developed a clinically significant loss of ascites during Amphenone administration.

D. Period IV, final prednisone: During this period, daily urine volume and electrolyte excretion tended to return to Period I values; however, patients who exhibited a marked sodium diuresis in Period II continued to lose sodium and water until ascites was no longer evident.

Frequently, the most striking natriuresis due to Amphenone occurred 4 to 5 days after the first dose, which was after the drug had been discontinued in most cases. In Case 10 the maximum response was observed on the fifth day while he was still receiving 6 Gm. of Amphenone daily (Fig. 2), indicating that the delayed natriuresis observed in the other patients was probably not due to abrupt discontinuance of Amphenone.

E. Period V, final control: Daily balance studies approximated those of Period I except that mercaptomerin induced a greater increase in water and electrolyte excretion than during Period I.

Aldosterone Excretion.—Aldosterone excretion was above normal during the control period in all 4 patients studied; however, most of these values did not exceed those found in normal subjects on a sodium-restricted diet (Table III).[30]

Prednisone caused no consistent change in total aldosterone excretion nor in the ratio of "free" to "conjugated" fractions. In Case 2 there was a fall in aldosterone excretion over a 3-day period while on prednisone (Fig. 5); however, this was not accompanied by an increase in urinary sodium. The

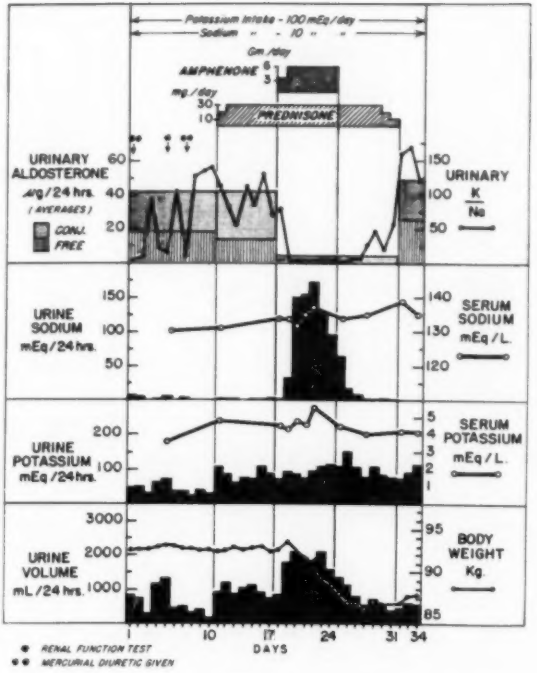

Fig. 2.

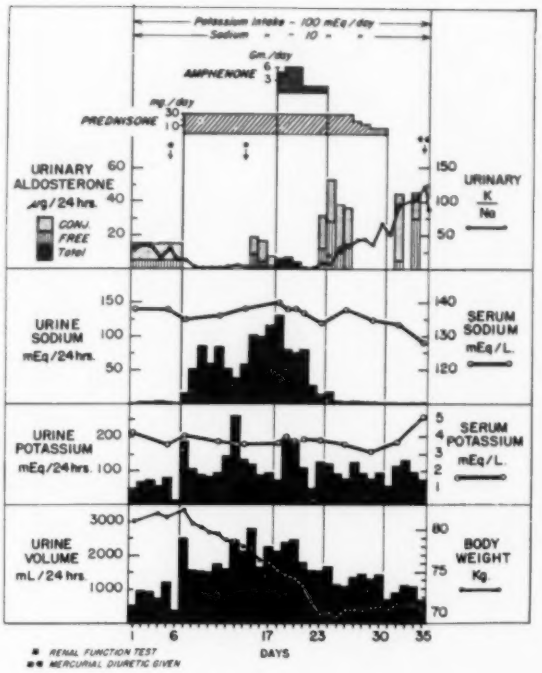

Fig. 3.

Fig. 2.—Balance study of a patient who showed a marked sodium diuresis in response to Amphenone administration. (W. W., a 48-year-old man with portal cirrhosis and ascites.)

Fig. 3.—Balance study of a patient who showed a marked sodium diuresis in response to prednisone alone, with no enhancement associated with the addition of Amphenone. He was clinically free of ascites by the end of the Amphenone period. (M. K., a 40-year-old man with portal cirrhosis and ascites.)

massive sodium diuresis exhibited by Case 9 in response to prednisone administration was not accompanied by a change in aldosterone excretion (Fig. 3). Thus, the natriuresis induced by prednisone in this case could not be related to a fall in aldosterone excretion.

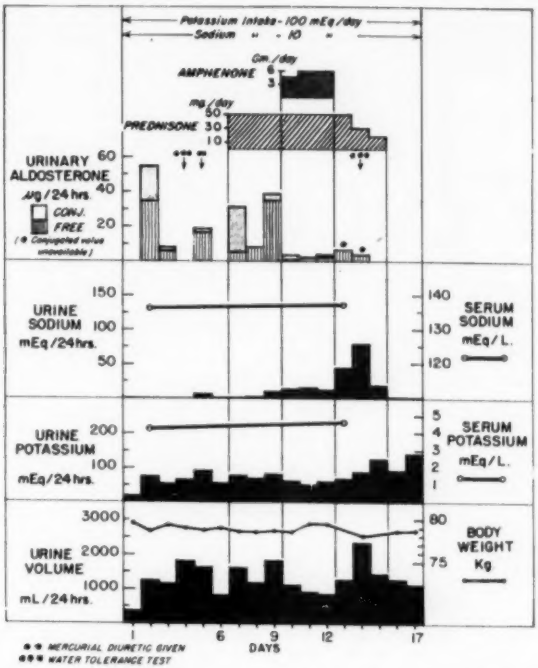

Fig. 4.—Balance study in a patient who showed a slight sodium diuresis in response to prednisone and Amphenone. (J. A., a 62-year-old man with portal cirrhosis and ascites.)

Amphenone administration caused a 60 to 90 per cent decrease in aldosterone excretion in all 4 patients in whom it was measured (Table III); however, this was not always associated with an increase in sodium excretion (Figs. 2 to 5). Case 2 exhibited no increase in sodium excretion despite a decrease in aldosterone excretion, and in Case 9 the decrease in aldosterone excretion following Amphenone actually was associated with a relative fall in a previously elevated sodium excretion (Fig. 3). An increased sodium excretion was associated with the fall in aldosterone excretion in Cases 1 and 10. However, in subsequent studies, Case 1 exhibited a diuretic response to prednisone alone. Thus, of the 4 patients so studied, Case 10 was the only one in whom the natriuresis could be definitely attributed to a decreased aldosterone effect.

The aldosterone excretion of patients without liver disease on an unrestricted diet is 0 to 15 µg per 24 hours with a mean of 5, using the method

TABLE IV. CHANGES IN SERUM CONSTITUENTS FOLLOWING PREDNISONE AND AMPHENONE ADMINISTRATION

PATIENT	SERUM SODIUM (mEq./L.)			SERUM POTASSIUM (mEq./L.)			SERUM CHLORIDE (mEq./L.)			BLOOD HEMATOCRIT (%)			SERUM ALBUMIN (GM. %)			SERUM BILIRUBIN (MG. %)		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1 (1)	136	—	137	4.3	—	4.6	94	—	99	37	—	—	4.0	—	3.6	1.6	—	0.1
(2)	124	136	—	5.5	5.3	—	93	90	—	37	38	—	2.8	2.8	—	2.3	1.9	—
3 (1)	136	—	131	4.6	—	5.8	97	—	103	25	—	32	3.7	—	3.7	1.2	—	0.8
(2)	138	139	—	4.0	4.0	—	105	99	—	26	30	—	3.8	4.1	—	—	—	—
2	138	138	139	4.0	4.3	4.5	92	98	101	—	—	—	4.6	—	4.7	0.8	—	0.4
4 (1)	136	132	—	4.0	5.7	—	96	99	—	31	31	—	4.2	—	—	5.1	3.4	—
(2)	129	136	—	3.6	4.4	—	97	100	—	27	30	—	3.1	2.8	—	3.4	3.7	—
7	135	136	132	4.2	5.4	5.4	90	97	92	31	29	28	2.1	2.5	2.6	0.2	0.2	0.1
8	132	138	137	3.4	3.9	3.8	99	119	103	32	33	35	3.2	2.9	2.8	2.0	1.3	1.2
5	127	138	—	4.9	3.8	—	95	105	—	33	35	—	2.8	3.6	—	2.6	1.3	—
6	142	140	—	3.8	3.8	—	95	106	—	34	36	—	2.8	3.0	—	2.3	1.5	—
9	135	139	137	4.1	3.6	3.8	106	102	104	36	36	35	1.9	3.1	—	4.3	3.3	3.5
10	131	134	134	4.8	4.5	4.5	102	102	109	30	34	33	3.6	—	3.9	1.8	1.5	1.5

Note: (a) 1 (2), 3 (2), and 4 (2) refer to a repeat study in the same patient. (b) I, II, and III refer to periods of study as described in the text.

described above (Table III). With sodium restriction, the aldosterone excretion rises to a level of 10 to 45 μg per 24 hours by the third to fourth day and subsequently may fall to a level slightly above initial values.[30] Marked individual variation is usually observed.

The mean "free" urine aldosterone ranged from 32 to 81 per cent of the total ("free" plus "conjugated"), which may be contrasted with a mean of 20 per cent for patients without liver disease (Table III). This value for normal subjects is in agreement with the results of Axelrod and associates[8] using a bio-assay technique. The ratio remained relatively constant in each patient throughout the study, although there was a trend toward normal in Case 2 during Periods III and IV.

Urine K to Na ratios,* calculated for Cases 9 and 10 (Figs. 2 and 3), could not be closely correlated with aldosterone excretion. This lack of correlation had been observed in normal subjects on unrestricted[24] and on sodium-restricted diets,[30] using the same method for aldosterone determination. This is in contrast with the experience of Luetscher and Curtis, using the bio-assay technique.[31]

Serum Electrolytes and Related Measurements.—The effect of prednisone and Amphenone on serum electrolytes and related measurements is seen in Table IV. During prednisone administration (Period II) the serum sodium concentration rose 7 to 12 mEq. per liter in 3 instances; 3 to 7 mEq. per liter in 3; and remained essentially unchanged in 4 (the latter in patients with relatively normal serum sodium concentrations). A fall of 4 mEq. per liter was noted in one instance, Case 4 (2). The 3 serum sodium concentrations between 124 and 130 mEq. per liter at the end of the control (Period I) were above 135 mEq. per liter after prednisone (Period II) (Fig. 6). A rise of serum sodium concentration began in Case 5 within 2 days, before changes in sodium or fluid balance were evident. With this data it is impossible to determine whether these changes in serum sodium concentration were related to an internal shift of fluid and electrolytes or to increased urine volume and increased insensible fluid loss. Serum solute concentration rose in all 6 patients so measured (Table II).

A corresponding but less significant rise occurred in total serum protein and hematocrit in response to prednisone administration. There were no consistent changes in nonprotein nitrogen or carbon dioxide content. Serum chloride concentration tended to rise during prednisone administration in all but 3 instances. Serum potassium concentration was normal in most instances, and there were no consistent deviations. Serum bilirubin concentration tended to fall slightly while serum albumin concentration tended to rise during prednisone administration. There was no correlation between pretreatment serum albumin or bilirubin concentrations and a response to prednisone, and improvement in these values was maintained through the final control period.

*See footnote on page 400.

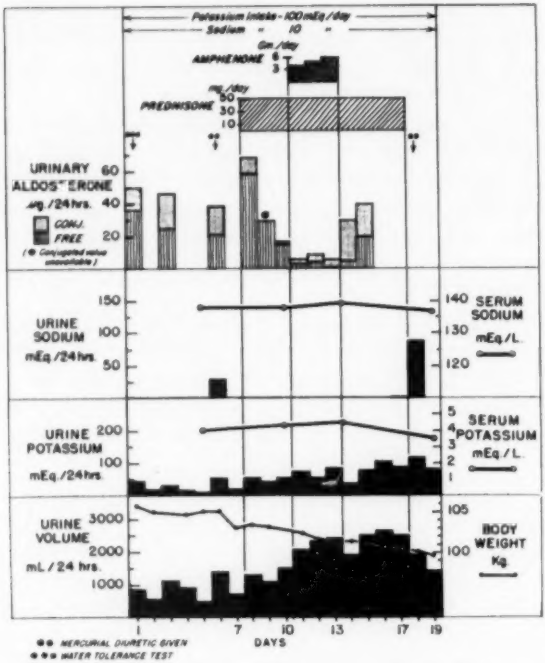

Fig. 5.—Balance study in a patient who showed no sodium diuresis in response to prednisone or Amphenone administration. (J. O., a 40-year-old man with portal cirrhosis and ascites.)

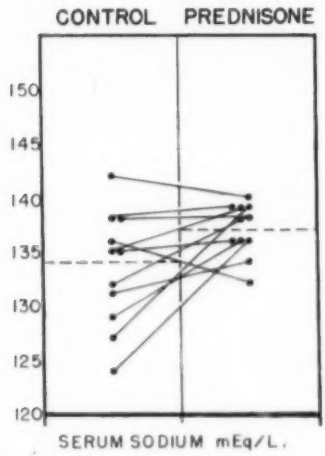

Fig. 6.

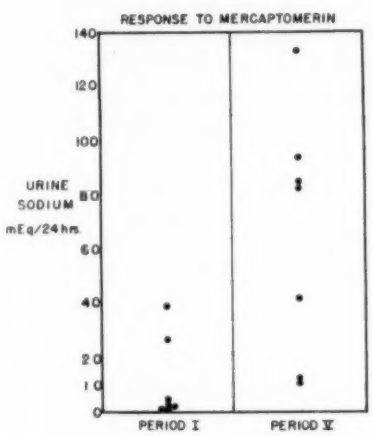

Fig. 7.

Fig. 6.—Change in serum sodium in response to prednisone administration. Eleven studies in 10 patients. (See Table IV.)

Fig. 7.—Urine sodium excretion in response to 2 ml. mercaptomerin subcutaneously before and after prednisone administration.

The administration of Amphenone for short periods produced no striking or consistent changes; however, 3 patients showed a slight rise in serum potassium and a fall in serum sodium. No significant alteration in serum albumin or bilirubin concentration or cephalin flocculation was noted.

Response to Mercurial Injections.—The majority of patients exhibited a poor response to mercaptomerin during the initial control period, although 3 (Cases 5, 6, and 8) excreted over 20 mEq. of sodium on the day of injection. A small increase in sodium excretion was seen on the day after injection in several instances.

Following discontinuance of prednisone (Period V), mercaptomerin administration increased urine sodium excretion from 2 to 100 times the Period I values (Fig. 7). This increased sensitivity usually decreased rapidly with repeated mercurial injections. In 3 patients (Cases 3, 4, and 7) who did not achieve a significant sodium diuresis on prednisone, the addition of mercurials in 2- or 3-day intervals for a period of 9 to 12 days, although transiently increasing urinary sodium, did not cause a significant weight loss or loss of ascites.

Hypochloremia and decreased glomerular filtration rate both may be factors in mercurial unresponsiveness.[32, 33] Steroids are known to increase the glomerular filtration rate, although this may be inconstant in patients with cirrhosis.[34] The serum chloride was higher during the final control period in each patient except Case 7; however, there was no consistent correlation between the amount of change in serum chloride levels and the degree of responsiveness to mercaptomerin.

Clinical Observations and Complications.—Coincident with prednisone administration, a feeling of well-being and euphoria was experienced by all patients; however, there were no serious psychologic complications. Appetite increased markedly in some patients. Complaints of between-meal hunger were treated with interval feedings subtracted from the daily diets and with aluminum hydroxide gel.* Antrenyl,†[35] an anticholinergic drug, was added in some instances. No evidence of peptic ulceration or of upper gastrointestinal hemorrhage was observed, although bleeding in small amounts from rectal hemorrhoids occurred in 3 patients. An increase in peripheral venous distention was noted sporadically, and a softening of the abdomen was noted occasionally in patients with tense ascites, prior to fluid balance changes.

Serious complications ending fatally developed in 2 instances. Both occurred following repeated courses of prednisone in severely debilitated patients who had clinical and laboratory evidence of far-advanced liver disease with recent episodes of impending hepatic coma. Both patients were severely ill with grave prognoses at the time of the repeated courses of prednisone, and it is felt that steroids masked the systemic response to infection making diagnosis difficult and may have potentiated the virulence of the infections by impairment of host resistance.

*Maalox, William H. Rorer, Inc., Philadelphia, Pa.

†We are indebted to Ciba Pharmaceuticals, Inc., Summit, N. J., for supplies of Antrenyl.

Amphenone produced a syndrome clinically indistinguishable from impending hepatic coma in the 2 patients in whom it was given alone (Cases 3 and 10). Toxicity within 1 to 4 hours after the initial dose forced discontinuance of the drug in each case. These symptoms were less severe and their onset was delayed until the second or third day when prednisone was given prior to and during Amphenone administration. Even on the combination of drugs, 6 of 7 patients developed symptoms of impending hepatic coma, while Case 10 developed no toxic symptoms after 6 days of prednisone (30 mg. per day), and Amphenone (6 Gm. per day). This reaction to Amphenone and its relation to hepatic function have been discussed in previous reports.[17, 22, 29] There was no evidence of aggravation of pre-existing liver disease as a result of prednisone or Amphenone administration.

DISCUSSION

The findings reported in this study supplement previous work showing that Amphenone inhibits the adrenal cortical biosynthesis of aldosterone[14, 16, 17, 19] and other steroids[14, 15, 17, 18] in man. Increased sodium excretion accompanied Amphenone administration in 5 of 7 patients to whom it was administered; however, sodium loss could be correlated definitely with an Amphenone effect in only 4. The fall in urine aldosterone (observed in all 4 patients in whom it was measured) during Amphenone administration was correlated with increased urine sodium in 2 instances, but this natriuresis could be attributed definitely to aldosterone inhibition in only one case. Toxicity prevented prolonged Amphenone administration, thus limiting prolonged study and clinical usefulness.

The elevated aldosterone excretion observed during the control period is in agreement with previous work with cirrhotic patients[6-8] and is consistent with the findings of increased aldosterone excretion in other edematous states.[6, 36, 37] Nevertheless, the demonstration that reduction of dietary sodium in normal subjects will cause an increased aldosterone excretion, followed several days later by a decrease,[30, 38, 39] makes interpretation of our results difficult in view of the prolonged strict dietary sodium restriction. Thus, although aldosterone excretion during the control period was elevated in all patients measured, it was often not above that seen in patients without liver disease on a sodium-restricted diet (Table III).[30]

A sodium diuresis occurred in response to prednisone alone in 5 of 10 patients, and was clinically significant in terms of loss of ascites in 3. Aldosterone excretion (measured in 4 patients with and without increased urine sodium) could not be correlated with the prednisone-induced natriuresis; in fact, a marked diuresis occurred over a 10-day period in one patient, while the aldosterone excretion remained unchanged. This finding is not in agreement with the work of Vesin and co-workers[40] who observed a marked fall in aldosterone excretion in conjunction with a prednisone diuresis in one patient, nor with that of Muller and associates[41] who observed a slight decrease in aldosterone excretion in a patient with cirrhosis receiving prednisone,

although he did attribute the diuresis to other causes as well as to aldosterone reduction. However, our findings are consistent with the observation that a prednisone-induced diuresis in a patient with nephrotic syndrome was not associated with a fall in urine aldosterone.[42]

The mechanisms controlling aldosterone secretion in man under physiologic conditions are poorly understood. Production of aldosterone appears to be controlled partly by changes in extracellular fluid volume,[43, 44] partly by blood potassium concentration,[31, 45, 46] and partly by adrenocorticotrophic hormone (ACTH),[47-49] although it is evident that not all changes in aldosterone excretion can be accounted for by these known mechanisms.[50, 51] No clear relationship is known between any one of these controlling factors and the increased aldosterone excretion observed in patients with cirrhosis and ascites. Thus, there is no increase in serum potassium.[3] Moreover, measurable extracellular fluid volume is not decreased, even on a sodium-restricted diet,[52] and expansion of the extracellular fluid by infusions does not consistently cause diuresis.[53] Aldosterone does not seem to be ordinarily regulated by ACTH,[47] and inhibition of ACTH with cortisone or prednisone has not produced a constant diuresis.[34, 54, 55] In addition, our results fail to show a consistent fall in aldosterone excretion in response to prednisone administration. The demonstration that urine aldosterone excretion increases following abdominal paracentesis in cirrhosis[7, 56] is in accord with the concept of aldosterone secretion regulated by fluid volume changes, in view of the observation that paracentesis is a "dehydrating" procedure in cirrhosis (i.e., a decrease in plasma and nonascitic extracellular fluid volumes).[11, 57] Thus, aldosterone secretion in cirrhosis may increase in response to a diminished "effective but unmeasurable" extracellular fluid volume or to diminished intravascular fluid volume which is secondary to the transudation of fluid into the abdominal cavity. The lack of marked increases in aldosterone excretion noted in our patients during control periods may reflect the small amount of ascites formation. Although our data suggest a relationship between aldosterone excretion and sodium retention in these patients, the absence of complete correlation would indicate that other factors also may be involved. Refinement in technique may be needed to elucidate the mechanism of increased aldosterone excretion in cirrhosis with ascites.

The present observations raise the question of whether the amounts of aldosterone measured in the urine actually reflect quantitatively the rate of secretion of the hormone by the adrenal cortex. As the amount of aldosterone excreted in the urine represents only a small fraction of adrenal production, the significance of observations based on urinary excretion is somewhat limited. Work with animals has shown that large increases in aldosterone as measured in the adrenal vein may not be reflected in the peripheral blood, and the amount appearing in the urine has been estimated to be as small as 1 per cent of adrenal secretion.[58] In man, using labeled aldosterone, the proportion of infused aldosterone excreted in urine was 4 per cent.[59] A previous discussion has emphasized the reliability of the analytic procedure used

in the present study[24]; however, it is possible that in patients with cirrhosis, the impaired liver function might allow a greater than normal proportion of adrenal aldosterone production to be excreted unaltered in the urine. Suggestive evidence that cirrhosis alters aldosterone metabolism arises from the increased proportion of urine aldosterone present in the free or readily hydrolyzable form. This is in accord with the results of Axelrod and associates[8] and would indicate that the metabolism of aldosterone and of cortisol do not necessarily follow similar pathways. Indeed, Englert and associates[60] showed that, although the reduction of cortisol was impaired in liver disease, the conjugation of its metabolites proceeded unimpaired. A more definite answer to these problems must await a satisfactory method of measuring plasma aldosterone.

In contrast to the variable effect of prednisone on aldosterone and sodium excretion, a uniform effect on urine volume was observed. All patients developed increased urine volume during prednisone administration, while only 5 of 10 developed increased urine sodium excretion, and this natriuresis was not directly related to the degree of increase in urine volume. These findings are consistent with the results of others.[34, 54, 55]

Continued administration of adrenal steroids to normal men influences the renal tubular reabsorption of water causing an increase in the maximal rate of water diuresis, which is unrelated to changes in glomerular filtration rate, solute excretion, or antidiuretic hormone effect.[61] A similar mechanism may be responsible for the diuretic effect of prednisone in cirrhosis with ascites. Endogenous creatinine clearance during prednisone administration[34] suggests that glomerular filtration rate alterations are not related to the diuresis of these patients. The increased urine volume did not seem to represent purely a solute diuresis, for the urine solute concentration in some patients receiving prednisone decreased, indicating that the rise in solute output was less than the rise in urine volume. The rise in serum solute concentration following prednisone administration is consistent with a decreased antidiuretic hormone effect; however, the inadequacy of this postulate is indicated by the finding of some daily urine solute concentrations of 400 to 500 mOsm. per liter and over.* In fact, the rise in serum solute concentration would be expected to stimulate secretion of antidiuretic hormone. Thus, the concept of an altered renal tubular reabsorption of water unrelated to changes in glomerular filtration, solute excretion, or antidiuretic hormone effect could also apply to the patients in this study. In acute studies on normal subjects, sodium diuresis invariably accompanied water diuresis due to adrenal steroid administration,[61] while only half of the patients with cirrhosis exhibited an increased sodium excretion. This is in accord with previous observations that patients with cirrhosis and ascites may retain sodium avidly even in the face of strong stimuli for sodium excretion. The data presented in this study are

*Antidiuretic hormone is necessary for a concentrated urine under ordinary circumstances; however, Berliner[62] has shown that a concentrated urine may be formed in the absence of antidiuretic hormone under certain conditions when there is a decreased volume of urine reaching the concentrating segments of the renal tubules.

insufficient to allow a final conclusion concerning the method by which prednisone affects water and sodium excretion in patients with cirrhosis and ascites; however, the data do not support the concept[40] that a decreased aldosterone excretion alone is involved in the natriuresis caused by prednisone.

Amphenone administered in small doses to patients with liver disease causes a syndrome clinically similar to impending hepatic coma, and the coadministration of prednisone was necessary to decrease the severity of this reaction. The serious complications noted in 2 patients following repeated courses of prednisone warrant the use of caution when administering this drug to severely debilitated patients. It should be emphasized that the patients in this study were selected for their lack of response to conventional diuretic measures, and many were seriously ill. At present, the adverse manifestations of prednisone and Amphenone are a serious limitation to their clinical effectiveness for this group of patients.

SUMMARY

1. Partial inhibition of adrenal steroid production by Amphenone in patients with cirrhosis and ascites, who were maintained on prednisone and sodium restriction, was associated with increased urine sodium excretion in 5 of 7 cases, although a clinically significant loss of ascites occurred in only one. A complete inverse correlation between sodium and aldosterone excretion was not observed, and it is suggested that other factors as well as aldosterone secretion are operative in causing the sodium retention of these patients. In addition, the data indicate that patients with cirrhosis may have an impaired ability to excrete conjugated aldosterone.

2. Prednisone was found consistently to cause a water diuresis when administered to patients with cirrhosis, and this was associated with an increased response to mercurial diuretics and frequently a rise in serum sodium. Increased sodium excretion followed prednisone administration in 5 of 10 cases but was clinically significant in only 3 and could not be correlated with decreased aldosterone excretion.

3. The adverse neurologic manifestations following Amphenone administration to patients with cirrhosis, even though ameliorated by concomitant prednisone administration, create a severe limitation to its clinical usefulness. Prolonged prednisone administration to these patients with cirrhosis was associated with overwhelming infection in 2 instances.

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ORNITHINE CARBAMYL TRANSFERASE IN DOG SERUM ON INTRAVENOUS INJECTION OF ENZYME, CHOLEDOCHUS LIGATION, AND CARBON TETRACHLORIDE POISONING

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IN THE mammal, ornithine carbamyl transferase (OCT)[1] has hitherto been found only in liver cells.[2] It occurs normally in minute amounts in blood serum and is often increased in liver disease. This rise may be of diagnostic and prognostic significance.[3] The aim of the present investigation was to elucidate some factors with an important bearing on the diagnostic evaluation of serum OCT.

MATERIAL AND METHODS

The experimental animals were all healthy mongrel dogs, weighing between 13.5 and 21 kilograms, that had been dewormed and vaccinated against distemper.

Operations.—All dogs were anesthetized with Nembutal 0.035 Gm. per kilogram of body weight intraperitoneally, and received 600,000 I. U. procaine penicillin by intramuscular injection to prevent infection. Intubation was done in order to obviate anoxia. The operations were performed by Dr. G. Jonson and Dr. D. Hallberg.

Ligation of the Common Bile Duct.—After opening the abdomen by a midline incision, the choledochus was identified, ligated, and then cut 3 to 5 cm. from the duodenal papilla. The belly was closed within 30 to 60 minutes. All dogs recovered consciousness within 6 hours after the onset of anesthesia, and were on their feet the day after operation.

At subsequent necropsy the bile duct obstruction was checked and found to be complete.

Control animals were subjected to the same operative procedure with the exception of ligation and division of the choledochus.

Reoperation.—The dogs that had undergone choledochus ligation were submitted, 15 to 17 days later, to a second operation at which time the continuity of the common bile duct was restored by means of a Teflon tube. This operation lasted from 2 to 3 hours and greatly impaired the general condition of the jaundiced animals. In 2 of them fluid had to be administered intravenously.

Carbon Tetrachloride Poisoning.—In 4 anesthetized dogs, 1 ml. of carbon tetrachloride per kilogram of body weight was instilled through a gastric tube. These animals recovered consciousness within 4 hours after the onset of anesthesia. Their condition had deteriorated and they were unwilling to get on their feet.

Preparation of Liver Extract Containing OCT.—The abdomen of anesthetized dogs was opened rapidly and 100 Gm. of liver tissue removed, then cooled with ice to 0° C. All further manipulations were done at that temperature. The liver tissue was homogenized, together with 300 ml. distilled water, in a Waring blendor. This treatment releases OCT from the mitochondria. The homogenate was centrifuged at 20,000 g for 30 minutes. The light-red slightly opalescent fluid was used within 24 hours. The extract had a high OCT activity (Table III).

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Tissue Homogenates.—Tissue homogenates of different organs were prepared from healthy dogs that had been killed by intravenous injection of air during anesthesia. All specimens were taken within 5 minutes after death and treated as above but not centrifuged.

OCT Activity.*—In serum or in tissue homogenates OCT activity was determined by incubation with citrulline-carbamyl-C^{14} in arsenate buffer.[4] The amount of $C^{14}O_2$ formed is proportional to the OCT activity. Results were expressed in micromoles of $C^{14}O_2$ per 0.5 ml. of serum or 1 Gm. of tissue.

Other Determinations.—Glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were assayed by the method of Reitman and Frankel.[5] Results were expressed in Karmen units.[6]

RESULTS

OCT Content.—The OCT content of different organ homogenates is detailed in Table I. No OCT activity exceeding that found in normal serum (0.024 ± 0.010 μM of $C^{14}O_2$ per 0.5 ml. of serum) was demonstrable in brain, lung, spleen, pancreas, kidney, striated muscle, or heart muscle. Gall bladder bile, stomach, cecum, and ileum showed enzyme activities equivalent, respectively, to 0.06, 0.74, 2.60, and 43.0 μM of $C^{14}O_2$ per 1 Gm. of tissue or per 1 ml. of bile. The corresponding value for liver was 2,900 μM of $C^{14}O_2$.

TABLE I. OCT CONTENT IN THE DIFFERENT ORGANS FROM DOG. ALL VALUES ARE THE MEANS OF DETERMINATIONS FROM 2 ANIMALS

ORGAN	OCT ACTIVITY IN μM $C^{14}O_2$ PER Gm. TISSUE
Brain	<0.01
Lung	
Spleen	
Pancreas	
Kidney	
Striated muscle	
Heart muscle	
Blood serum	0.04
Gall bladder bile	0.06
Stomach (pyloric portion)	0.74
Duodenum	36.0
Ileum	43.0
Cecum	2.6
Liver	2,900.0

To rule out intestinal contents as the source of OCT activity, both washed and unwashed specimens from stomach, ileum, and cecum were incubated, but no verifiable difference in enzyme activity emerged.

OCT Levels.—The 24-hour OCT levels in dog serum are set forth in Table II. In addition to OCT, both GOT and GPT were assayed since they were consistently used as reference enzymes. Blood samples were taken here, as in all experiments, by puncture of a leg vein. No 24-hour variation of significance was observed.

Elimination Rate of Liver Extract.—The elimination rate of intravenously administered liver extract containing OCT is shown in Table III. The mean for

*In an earlier paper[4] the concentration of citrulline-carbamyl-C^{14} was erroneously given as 1 μM per milliliter. This should be 10 μM per milliliter of final incubation mixture.

the 3 dogs will be found in Fig. 1. The 3 different liver extracts had about the same OCT content (660 to 730 μM of $C^{14}O_2$ per 1 ml.). Their GOT and GPT contents, which were not determined, probably varied substantially.

TABLE II. THE 24-HOUR SERUM LEVELS OF OCT, GOT, AND GPT IN 2 DOGS. NORMAL VALUES: OCT, 0.024 ± 0.010 μM OF $C^{14}O_2$ PER 0.5 ML. SERUM; GOT, 18.5 ± 10.4 UNITS; GPT, 23.3 ± 10.0 UNITS. ALL NORMAL VALUES ARE FROM 15 DOGS

DOG NO.	DAY	TIME	OCT IN μM $C^{14}O_2$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
1	1	9:00 A.M.	0.011	20	16
		1:00 P.M.	0.018	30	25
		5:00 P.M.	0.018	25	16
		9:00 P.M.	0.010	25	15
	2	2:00 A.M.	0.018	25	16
		9:00 A.M.	0.018	30	20
2	1	9:00 A.M.	0.039	28	15
		1:00 P.M.	0.030	25	20
		5:00 P.M.	0.029	25	20
		9:00 P.M.	0.040	23	22
	2	2:00 A.M.	0.040	25	22
		9:00 A.M.	0.044	25	20

TABLE III. SERUM LEVELS OF OCT, GOT, AND GPT AFTER INTRAVENOUS INJECTION OF LIVER EXTRACT. THE LIVER EXTRACTS ADMINISTERED IN DOGS 3, 4, AND 5 HAD AN OCT ACTIVITY EQUIVALENT TO, RESPECTIVELY, 680, 660, AND 730 μM OF $C^{14}O_2$ PER MILLILITER. ALL ANIMALS WERE INJECTED WITH 2 ML. EXTRACT PER KILOGRAM BODY WEIGHT ON DAY 1 AT 8:00 A.M. IMMEDIATELY AFTER THE FIRST SAMPLE HAD BEEN TAKEN

DOG NO.	DAY	TIME	OCT IN μM $C^{14}O_2$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
3	1	8:00 A.M.	0.018	25	40
		10:00 A.M.	7.60	700	400
	2	10:00 A.M.	0.88	40	130
	3	10:00 A.M.	0.119	22	78
	4	10:00 A.M.	0.070	28	72
	6	10:00 A.M.	0.011	12	29
4	1	8:00 A.M.	0.038	26	32
		10:00 A.M.	8.30	1,300	1,600
	2	10:00 A.M.	0.79	90	1,000
	3	10:00 A.M.	0.370	18	715
	4	10:00 A.M.	0.044	12	440
	6	10:00 A.M.	0.037	25	160
5	1	8:00 A.M.	0.036	51	38
		10:00 A.M.	11.7	700	1,070
	2	10:00 A.M.	2.60	85	630
	3	10:00 A.M.	0.420	28	450
	4	10:00 A.M.	0.162	60	240
	6	10:00 A.M.	0.035	90	190

OCT was so eliminated from the blood stream that its serum level, measured 1, 2, and 3 days after injection of the liver extract, amounted to 14, 3, and 1 per cent, respectively, of the value determined 2 hours after the injection. The corresponding values for GPT were 59, 42, and 23 per cent, respectively. Serum GOT had fallen to 8 per cent after one day and was normal on the second day.

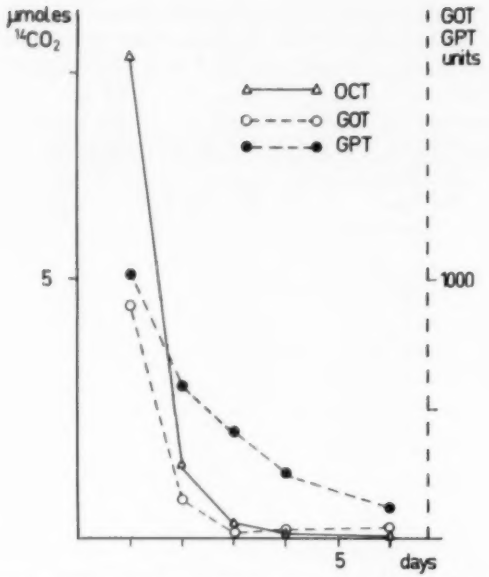

Fig. 1.—Elimination of intravenously administered liver extract on Day 1 (2 ml. per kilogram body weight). Means in 3 dogs from Table III. GOT and GPT in Karmen units.

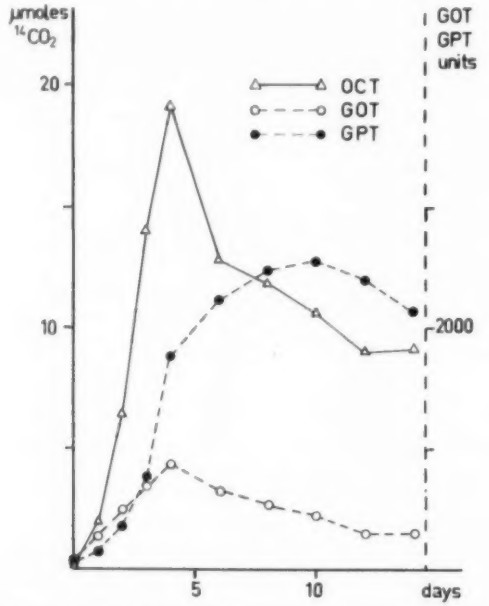

Fig. 2.—Serum OCT, GOT, and GPT after ligation of common bile duct (Day 0). Means in 3 dogs from Table IV. GOT and GPT in Karmen units.

OCT Content of Serum Following Choledochus Ligation.—The OCT of serum following choledochus ligation and in the controls is given in Table IV, the mean for the ligated dogs in Fig. 2.

TABLE IV. SERUM LEVELS OF OCT, GOT, AND GPT AFTER LIGATION OF CHOLEDOCHUS (DOGS 6, 8, AND 10) AND IN CONTROL (DOGS 7, 9, AND 11). THE OPERATIONS WERE PERFORMED ON DAY 0 BETWEEN 1:00 AND 5:00 P.M. SAMPLES WERE TAKEN AT 10:00 A.M. EACH DAY

DOG NO.	DAY	OCT IN μM $C^{14}O_2$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)
6	0	0.010	44	33
	1	0.238	150	45
	2	5.60	400	450
	3	10.2	520	1,250
	4	14.3	840	2,800
	6	13.5	400	4,000
	8	10.5	300	3,350
	10	8.40	200	3,350
	12	6.70	180	2,400
	14	8.60	170	2,400
8	0	0.045	42	30
	1	0.240	48	40
	2	4.00	110	110
	3	12.8	450	560
	4	23.0	680	1,100
	6	11.2	800	1,200
	8	10.5	580	*
	10	9.00	300	1,100
	12	8.60	250	1,250
	14	12.1	460	1,000
10	0	0.018	25	5
	1	5.50	650	380
	2	9.70	975	510
	3	18.8	1,075	1,100
	4	20.0	1,075	1,350
	6	13.8	750	1,500
	8	15.7	750	2,880
	10	14.3	850	3,200
	12	11.7	475	3,500
	14	6.70	305	2,000
7	0	0.020	42	18
	1	0.040	73	95
	2	0.072	104	62
	3	0.020	82	42
	4	0.014	37	42
	6	0.011	56	40
	8	0.015	42	24
9	0	0.011	35	12
	1	0.024	44	28
	2	0.010	32	15
	3	0.015	40	27
	5	0.009	50	28
	9	0.012	47	40
11	0	0.036	37	42
	1	0.570	70	180
	2	0.048	22	42
	3	0.043	32	95
	4	0.124	37	60
	6	0.065	25	55
	11	0.047	43	60

*Accidentally lost.

The controls exhibited little or no elevation of the enzyme level for the first few days after the operation. In the ligated group there were relatively slight rises on the first day. OCT reached a peak after 4 days, with a serum level 1,000 times normal, then gradually declined and, on the fourteenth day, amounted to 500 times normal. The GOT content rose to its maximum coincident with the OCT peak, when it was 30 times normal; then fell to about 10 times normal on the fourteenth day. The highest GPT values were found between the sixth and twelfth day, being around 150 times normal; the values then fell gradually to just under 100 times normal on the fifteenth day.

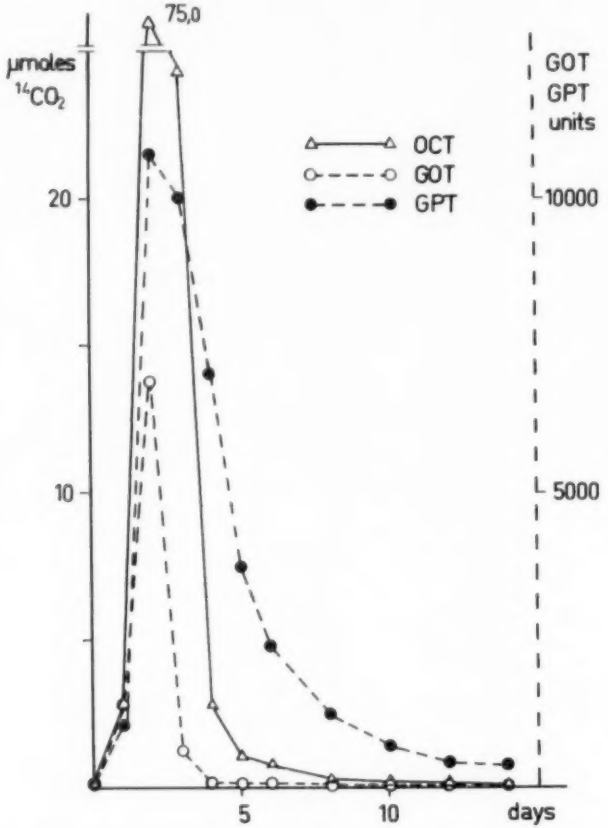

Fig. 3.—Serum OCT, GOT, and GPT after carbon tetrachloride poisoning (1 ml. per kilogram body weight) on Day 0. Means in 2 surviving dogs from Table VI. (GOT and GPT in Karmen units.)

In the 3 dogs with ligated choledochus the bile flow was restored by reoperation after 15, 17, and 15 days, respectively. The enzyme values are shown in Table V. Dog 6 died of biliary peritonitis 2 days after the second operation; Dogs 8 and 10 survived for more than 2 weeks.

TABLE V. SERUM LEVELS OF OCT, GOT, AND GPT IN DOGS IN WHICH THE BILE FLOW WAS RESTORED AFTER CHOLEDOCHUS LIGATION. DAYS ARE INDICATED WITH REFERENCE TO THE TIME OF THE FIRST OPERATION (DAY 0). THE SECOND OPERATION WAS PERFORMED BETWEEN 3:00 AND 9:00 P.M. BLOOD SAMPLES WERE TAKEN AT 10:00 A.M. THE DAY BEFORE AND ON EACH OF 3 DAYS AFTER OPERATION

DOG NO.	DAY	OCT IN μM $CitO_2$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)	REMARKS
6	14	8.60	170	2,400	
	15				Operation
	16	1.12	350	1,000	
	17	0.400	200	715	
	18				Dead
8	16	3.90	150	800	
	17				Operation
	18	2.60	180	1,440	
	19	0.250	20	500	
	20	0.070	26	580	
10	14	6.70	305	2,000	
	15				Operation
	16	1.92	120	1,500	
	17	2.28	120	1,050	
	18	2.11	130	930	

In 2 of the dogs all enzymes showed a reduction postoperatively; 1 dog (10) still had elevated OCT values, but the other serum enzymes tended to fall.

Serum OCT in Carbon Tetrachloride Poisoning.—The enzyme values are set forth in Table VI and the mean for the 2 surviving dogs in Fig. 3.

In all animals that survived, the maximum serum enzyme level was found on the second day. OCT, GOT, and GPT reached, respectively, 4,000, 200, and 400 times the normal value. Both OCT and GOT then fell rapidly; GOT showed normal values on the tenth day but OCT was still slightly elevated after 14 days, when GPT was 10 times normal.

DISCUSSION

Earlier it has been found[2] that in mammals OCT occurs only in the liver. According to my results, this is not altogether correct; in dogs, very small amounts are found in the stomach, bile, and cecum and somewhat greater amounts in the small intestine. Here, the content per unit weight is 1.5 per cent of that in the liver. A similar OCT distribution occurs in human organs.[7] The discrepancy between my results and earlier ones is in all probability due to the more sensitive methods used in the present investigation.

The important question arises as to whether extrahepatic OCT might influence its level in the serum, thus complicating the use of OCT in the diagnosis of liver disease. However, the amounts in the stomach and cecum are so minute that they probably cannot influence the serum level appreciably. Accordingly, elevated OCT values are not found in peptic ulcer and ulcerative colitis.[7] In the small intestine the OCT content is low, although not negligible, but diseases of that organ are not common. Therefore, elevated serum OCT values should point to liver damage if widespread disease of the small intestine can be ruled out.

About 85 per cent of the OCT (liver extract) injected intravenously in healthy dogs was eliminated in the first 24 hours. Elimination of endogenous OCT proceeded at the same rate in the dogs with carbon tetrachloride poisoning (Table VI) and in one dog with ligated choledochus in which the bile flow was restored (No. 8, Table V).

TABLE VI. SERUM OCT, GOT, AND GPT VALUES AFTER CARBON TETRACHLORIDE POISONING. ONE MILLILITER CARBON TETRACHLORIDE PER KILOGRAM BODY WEIGHT WAS ADMINISTERED THROUGH A GASTRIC TUBE AT 11:00 A.M. ON DAY 0. BLOOD SAMPLES WERE TAKEN AT 10:00 A.M. EACH DAY

DOG NO.	DAY	OCT IN μM $C^{14}O_2$ PER 0.5 ML. SERUM	GOT (KARMEN UNITS)	GPT (KARMEN UNITS)	REMARKS
12	0	0.012	12	29	
	1	4.50	2,400	1,000	
	2	57.0	10,000	12,000	
	3	7.60	1,000	*	
	4	1.80	120	10,000	
	5	0.840	80	4,500	
	6	0.630	94	3,000	
	8	0.223	43	1,700	
	10	0.195	32	960	
	12	0.128	38	500	
	14	0.056	28	500	
13	0	0.018	24	30	
	1	12.0	4,200	4,600	
	2	62.0	6,750	11,000	
	3	2.40	850	3,000	
	4				Dead
14	0	0.030	25	32	
	1	1.24	400	1,100	
	2	92.0	3,700	9,500	
	3	41.0	240	9,600	
	4	3.70	80	3,900	
	5	1.28	77	2,900	
	6	1.04	56	1,860	
	8	0.320	47	800	
	10	0.095	24	400	
	12	0.090	18	300	
	14	0.110	25	245	
15	0	0.028	22	10	
	1	109.0	22,500	18,690	
	2				Dead

*Accidentally lost.

A similar rapid elimination of OCT also occurs in human beings, as shown in patients with gall stone colic, where the usual substantial rise of OCT subsides on the second day.[7]

Therefore elevated serum values indicate that biliary stasis or acute liver damage either is present or has been present and relieved not more than 2 days before the test.

After ligation of the common bile duct an initial delay of the OCT rise in serum appears. This delay is less marked when the gall bladder has been removed.[8] This different behavior is most readily explained by the pressure-equalizing effect of the gall bladder that is known to influence the serum bilirubin level in a similar way.

The dogs with carbon tetrachloride poisoning showed, on the second day, excessive serum enzyme concentrations, which for OCT amounted to 4,000 times the normal value. After this huge OCT rise the serum values fell rapidly, thus suggesting that the damaged liver cells discharged their OCT content quickly.

If the findings in dogs are applicable to man, determination of OCT could be useful in the diagnosis of liver disease. In massive necrosis of the liver, the serum OCT should rise quickly and then rapidly fall. Total occlusion of the choledochus, on the other hand, should be associated with rising serum OCT values for a few days and then a gradual fall during the ensuing weeks.

Clinical obstruction of the choledochus is often partial and few patients with this condition can be expected to show the OCT behavior typical for choledochus ligation in dogs. Here, as in moderate and mild cases of hepatitis, further clinical studies in human subjects will have to decide if determination of serum OCT may be of diagnostic aid.

SUMMARY

Ornithine carbamyl transferase (OCT) does not normally occur in the canine brain, lung, spleen, pancreas, kidney, striated muscle, or heart muscle. Minutes amounts are found in bile, stomach, and large intestine. The small intestine contains per unit weight about 1.5 per cent of the OCT content of the liver.

In dogs, about 85 per cent of intravenously injected OCT disappears within one day and the rest within 6 days. In choledochus ligation, OCT rises rather slowly in serum the first day, then much faster, and reaches its peak on the fourth day with a serum level 1,000 times normal. The OCT serum level is still 500 times normal after 14 days. In severe carbon tetrachloride poisoning, maximum OCT values appear in serum on the second day, with 4,000 times normal. Thereafter, the OCT level sinks rapidly and is only very slightly elevated after 14 days.

I wish to thank Professor Henrik Lagerlöf for helpful discussions and valuable criticism. I am also indebted to Dr. Åke Senning for help and advice in connection with the dog experiments.

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THE BILE ACIDS OF MAN IN RELATION TO ATHEROSCLEROSIS

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CURRENT thinking implicates cholesterol in the pathogenesis of atherosclerosis. Whether the fault in cholesterol metabolism lies on the anabolic or catabolic side is not clear. Most experimental work has been concerned with the mechanism of synthesis or with variations in serum cholesterol, while relatively little attention has been given to the disposition of this steroid. In addition, most studies have been confined to animals.

Bloch and associates[1] found cholesterol to be converted to cholic acid in the dog, while experiments in a single human with a bile fistula resulted in recovery of part of the administered cholesterol as glycocholic acid in the biliary drainage.[2]

Wootton and Wiggins[3] studied the gall bladder bile acid distribution in a small number of autopsied cases, and concluded that there was no discernible correlation of composition with disease. Isaksson[4] examined human bile obtained at operation from "normal" gall bladders as well as from those associated with cholesterol stones. He found no correlation between age or sex and the ratios of the bile acids in normal bile, but suggested that both chenodesoxycholic and cholic acid concentrations are lowered in bile taken from males with cholelithiasis.

Studies by Abell and associates[5] in the dog have established a correlation between diet, thyroid function, serum cholesterol, atherosclerosis, and the excretion of bile acids. The present work was undertaken as a preliminary attempt to extend these observations to man. In a series of 50 autopsied cases, a definite correlation was found between the ratio of DCA to TCA,† and the degree of atherosclerosis.

METHODS AND MATERIALS

Gall bladder bile was obtained immediately post mortem from 50 patients who had died from a variety of conditions. The specimens were obtained consecutively except when technical difficulties caused exclusion of a patient. Bile was held at −20° C. Storage for 24 hours at 4° C. resulted in no distortion of DCA/TCA ratios, nor did storage for several months at −20° C. The DCA and TCA concentrations of the bile were assayed by the method

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†Abbreviations used: DCA = dihydroxycholanic acid(s); TCA = trihydroxycholanic acid(s).

of Mosbach and associates[6] using pure bile acids* as reference standards. Each specimen was analyzed in triplicate. Occasional samples were checked by separation of the acids on a celite column.[7] Results are reported in terms of milligrams per gram of liquid bile, since the viscosity of concentrated bile made pipetting inaccurate.

Postmortem examinations were performed by the Department of Pathology. Special attention was given to the aorta, coronary arteries, and adrenal cortex. While cerebral and peripheral vessels were examined occasionally, insufficient evidence was accumulated to warrant tabulation. Atherosclerosis was graded as "none to slight," "moderate," or "advanced" depending on whether less than 10, 10 to 50, or more than 50 per cent of the vessel surfaces examined were involved. Few specimens were obtained from patients with cholelithiasis, partly because of the necessary exclusion of patients who had undergone cholecystectomy.

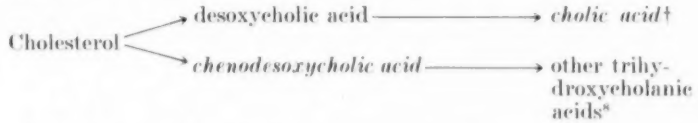
RESULTS

Twenty-two men, 17 women, and 11 children were examined. These had died from various causes, as indicated in Table I. Although there was rather marked variation among the individual cases, the patients with more marked atherosclerosis generally showed greater DCA to TCA ratios. This relationship is shown more strikingly in Table II, where the average values for the bile acids are tabulated for each of the grades of atherosclerosis considered.

When the data are considered in the light of the ages and sexes of the subjects (Table III), it may be seen that there is also an increase in the DCA/TCA ratio with age, a higher value being reached earlier in the male. Whether or not this is directly related to the increased incidence of atherosclerosis generally noted with age remains to be seen.

DISCUSSION

The details of bile acid formation are not complete. Data from animals plus a small amount of information from human experiments indicate that in certain species, such as the rabbit, which are more prone to develop atherosclerosis on cholesterol feeding, the chief end product of cholesterol metabolism is a dihydroxycholanic (desoxycholic) acid, while more resistant species such as the rat dispose of cholesterol chiefly as trihydroxycholanic (cholic) acid. The rat has been the most extensively studied laboratory animal with respect to bile acid metabolism. Degradation of cholesterol in the rat is thought at the present time to proceed according to the following scheme, the italicized acids being the principal end products recovered from bile.



In the normal rat the major pathway of cholesterol degradation is that leading to bile acids. Oxidation of desoxycholic acid to cholic acid occurs readily so that about 90 per cent of the total bile acid excreted is cholic acid.

*We are grateful to the Ames Company, Inc., Elkhart, Ind., for a gift of pure desoxycholic and cholic acids.

†While the name of the free bile acid is given, it is to be understood that either the glycine or taurine conjugate or both are implied.

TABLE I

PATIENT	AGE (YEARS)	DEGREE OF ATHERO-SCLEROSIS	DCA (MG./GM. OF BILE)	TCA (MG./GM. OF BILE)	DCA/TCA	DIAGNOSIS	NOTES
Men							
1	89	Advanced	4.01	1.66	2.42	Carcinoma of bladder	Uremia, pyelonephritis
2	87	Moderate	2.86	1.46	1.96	Infarct of jejunum	Old cerebral infarct, pyelonephritis, BUN 67
3	83	Advanced	4.20	2.62	1.60	Myocardial infarct	Ill 10 days, terminal uremia
4	83	Advanced	3.72	1.55	2.40	Acute myocardial infarction	Fairly recent prostatectomy
5	80	Advanced	8.12	3.95	2.06	Myocardial infarct	Cholelithiasis
6	76	Slight	0.98	0.82	1.18	Benign prostatic hyperplasia	Hydronephrosis, N.P.N. 240
7	75	Slight	17.66	7.84	2.25	Carcinoma of stomach	Liver metastases
8	74	Slight	19.91	20.56	0.97	Calcific aortic stenosis	Congestive failure, elevated B.U.N.
9	73	Advanced	30.87	11.94	2.59	Chronic myelogenous leukemia	Uric acid 7.8
10	57	Advanced	40.04	18.18	2.20	Ruptured abdominal aneurysm	Postoperative death, hemolysis following transfusion
11	57	Slight	13.20	11.67	1.13	Friedlander's pneumonia	Slight cholesterolosis uremia, jaundice
12	56	Slight	1.02	1.67	0.61	Peritonitis	Slight portal cirrhosis
13	53	Slight	13.25	16.98	0.78	Portal cirrhosis	Jaundice
14	51	Advanced	11.68	4.08	2.86	Carcinoma of tongue	Old coronary occlusion with myocardial fibrosis and hypertrophy
15	48	Slight	6.31	1.53	4.12	Lymphosarcoma	Massive liver metastases
16	48	Slight	10.14	15.95	0.64	Malignancy of eye	Metastases
17	43	Slight	7.20	2.59	2.78	Portal cirrhosis	Bleeding varices, jaundice
18	41	Moderate	30.63	6.92	4.43	Arteriolar nephrosclerosis	Severe hypertension, uremia, enlarged heart, cerebral infarct
19	38	None	24.23	12.15	1.99	Mitral stenosis	Prolonged decompensation prior to death

20	31	Slight	25.38	17.88	1.42	Carcinoma of testis	Widespread metastases
21	27	Slight	11.59	4.92	2.36	Chronic myelogenous leukemia	
22	26	Moderate	20.78	18.09	1.15	Malignant pheochromocytoma	Cardiac hypertrophy
Averages	58.9		13.9	8.4	1.99		
Women							
23	80	Advanced	29.67	14.29	2.08	Carcinoma of colon	Recent cerebral infarct
24	77	Advanced	3.74	1.97	1.90	Carcinoma of vulva	Widespread metastases
25	75	Moderate	3.25	2.62	1.24	Esophageal ulcer	Died of empyema 4 weeks postoperative
26	73	Moderate	0.24	0.74	0.33	Carcinoma of lung	Widespread metastases
27	72	Slight	14.05	5.87	2.39	Tetanus	
28	70	Moderate	0.96	8.00	0.12	Arteriolar nephrosclerosis	B.U.N. 145, biliary cirrhosis—many gall stones
29	69	Advanced	3.69	1.69	2.18	Myocardial infarct	Brief final illness
30	63	Moderate	6.80	1.53	4.44	Carcinoma of ovary with metastases	Mild diabetes many years. B.U.N. 130, Na 109, Cl 77, K 5.6
31	63	Moderate	9.20	3.85	2.39	Carcinoma of stomach	Earlier cholesterol 206
32	52	Moderate	9.20	8.57	1.07	Carcinoma of thyroid	Local metastases. No hyperthyroidism
33	48	Moderate	11.88	7.41	1.60	Atrial septal defect	Postoperative death terminal uremia. B.P. 240/110
34	48	Slight	12.29	8.63	1.42	Mitral stenosis	Pulmonary emboli
35	46	Slight	17.26	10.73	1.61	Carcinoma of cervix	Long illness. B.U.N. 235 terminally
36	42	Moderate	14.25	5.25	2.71	Mitral stenosis	Died following commissurotomy
37	38	Slight	10.17	5.65	1.80	Mitral stenosis	Died following commissurotomy
38	29	Slight	1.81	4.49	0.40	Leukemia	
39	28	Slight	3.62	1.97	1.83	Myelofibrosis	Long illness, staphylococcus septicemia
Averages	57.2		8.4	5.5	1.74		

TABLE I—CONT'D

PATIENT	AGE (YEARS)	DEGREE OF ATHERO-SCLEROSIS	DCA (MG./GM. OF BILE)	TCA (MG./GM. OF BILE)	DCA/TCA	DIAGNOSIS	NOTES
Children							
40	19	None	29.43	27.31	1.08	Posttraumatic edema of brain	Auto accident day of death
41	16	Slight	1.72	1.05	1.64	Atrial septal defect	Postoperative death hydronephrosis
42	14	None	13.11	6.90	1.90	Chronic glomerulonephritis	Hypertension, uremia
43	10	Slight	19.25	16.35	1.18	Cerebral trauma	Died 2 days after auto accident
44	2	None	0.72	0.83	0.87	Tuberculous meningitis	
45	4 mo.	None	1.97	4.83	0.41	Meningitis	
46	8 days	None	23.77	19.70	1.21	Tracheo-esophageal fistula	
47	5 days	None	7.91	16.68	0.47	Tracheo-esophageal fistula	
48	4 days	None	1.93	1.36	1.42	Hydronephrosis	Septicemia and deep jaundice
49	3 days	None	10.23	4.50	2.27	Prematurity	
50	2 days	None	4.30	1.98	2.17	Prematurity	
Averages	5.5		10.4	9.2	1.33		

TABLE II. CHEMICAL CONSTITUENTS OF GALL BLADDER BILE IN PATIENTS GROUPED ACCORDING TO THE DEGREE OF ATHEROSCLEROSIS OBSERVED AT AUTOPSY

	DEGREE OF ATHEROSCLEROSIS			
	NONE TO SLIGHT (29 CASES)	MODERATE (11 CASES)	ADVANCED (10 CASES)	TOTAL (50 CASES)
DCA (mg./Gm.)	11.2	10.0	15.0	11.6
TCA (mg./Gm.)	8.7	5.9	6.2	7.5
DCA/TCA				
Total group	1.53	1.95	2.23	1.76
Adults	1.66	1.95	2.23	1.87
Children	1.33	----	----	1.33

Level of significance of DCA/TCA ratios in slight versus advanced atherosclerosis: for the total group $p = 0.02$; for adults only $0.05>p>0.02$. No significant difference noted between the ratios for children and adults in the group showing slight atherosclerosis.

TABLE III. CHEMICAL CONSTITUENTS OF GALL BLADDER BILE IN PATIENTS GROUPED ACCORDING TO AGE AND SEX

AGE GROUP	0 TO 19	20 TO 59		60 TO 89	
		M	F	M	F
Number of patients	11	13	8	9	9
Average age	5.5	44.3	41.4	80.0	71.3
DCA (mg./Gm.)	10.4	16.6	8.8	10.3	8.0
TCA (mg./Gm.)	9.2	10.2	6.6	5.8	4.5
DCA/TCA	1.33	2.04	1.57	1.94	1.90

Under special circumstances (e.g., hyperthyroidism) the secondary pathway assumes increased importance, and chenodesoxycholic acid constitutes more than 50 per cent of the end product.

The process of bile acid excretion in man is less well understood than is that of the rat but may resemble the scheme shown above. In man also chenodesoxycholic rather than desoxycholic acid has been shown to be the predominant doubly-hydroxylated acid.[3, 4] The pathways discussed relate to fresh gall bladder or fistula bile which has not been subjected to prolonged attack by intestinal bacteria.

The present study included patients with a variety of chronic diseases, and many of the patients included in this group had limited diets during the latter days of their hospitalization. It was not within the scope of this investigation to evaluate the effects of diet upon bile acid metabolism. In this connection, however, it may be noted that Mosbach and Kendall[9] have reported elevation of the DCA/TCA ratio in dogs given an "atherogenic" diet. This result coincides with that of the present investigation.

Since the method employed in the present study does not distinguish between chenodesoxycholic and desoxycholic acids, the complete significance of the results reported here is presently in doubt. Several alternate possibilities suggest themselves, depending upon which of the dihydroxycholanic acids predominates in the bile from patients with atherosclerosis, and work is in progress to determine the ratio of chenodesoxycholic to desoxycholic acid. The observations are also being extended to living patients, from whom more than one

specimen of bile may be obtained. At present all that can be said is that with increasing atherosclerosis there seems to be an increase in the ratio of dihydroxycholanic acid to trihydroxycholanic acid in gall bladder bile.

The present study did not, of course, permit analysis of more than one bile specimen from each patient, and additional samples of bile might well have shown some variability in DCA/TCA ratios. In this connection it might be noted that analysis of fresh daily specimens of fistula bile obtained following cholecystectomy from 4 patients have shown moderate day-to-day variations in the DCA/TCA ratio with the extreme limits for all 4 patients falling between 0.20 and 3.73.* Detailed observations on these patients will be the subject of a future report. It should be noted that the absolute values for bile acids reported in this study are of much less significance than the ratios because of the great variability in concentration of gall bladder bile. To perform balance studies by any simply designed experiment involving diversion of bile acids from the intestine is very difficult, since such diversion brings about profound alterations in the total bile acid and lipid metabolism.[10] Such studies in a human patient would require an Alexis St. Martin of elaborate design and extreme patience.

SUMMARY

1. Fifty patients were examined at autopsy with specific reference to arterial atherosclerosis, and to the bile acid content of gall bladder bile.

2. A significant correlation between degree of atherosclerosis and the ratio of dihydroxycholanic to trihydroxycholanic acids was noted.

3. Men in the 20 to 59 years of age group showed a higher ratio of dihydroxycholanic to trihydroxycholanic acids than did women in the same age group.

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*Compare references 4 and 6, in which average DCA/TCA ratios of 1.3 and 1.2, respectively, were observed.

THE INFLUENCE OF ALCOHOL AND DIETARY MAGNESIUM UPON HYPERCHOLESTEROLEMIA AND ATHEROGENESIS IN THE RAT

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With the Technical Assistance of M. Hazel, R. Cavanagh, P. Colon, and P. Connors

MANY clinicians are proponents of the viewpoint that chronic alcoholic patients have less atherosclerosis than do nonalcoholic patients.[1-5] Wilens[2] has suggested that this apparent difference may be explained in part by a decreased food intake, but to date only a few experiments have attempted to assess the role of alcohol in the pathogenesis of atherosclerosis,[6-8] and these studies have not shown a consistent relationship between alcohol and atherogenesis.

Eberhard[6] demonstrated that alcohol markedly increased serum cholesterol levels in the rabbit. This observation was not confirmed by Feller and Huff,[8] who reported that alcohol did not significantly alter serum cholesterol levels. Furthermore, these authors were unable to demonstrate an effect of alcohol upon atherogenesis in rabbits.

Flink[9] found decreased serum magnesium levels in chronic alcoholic patients while Klingman and associates[10] observed an increased tendency toward convulsions in rats fed alcohol and low-magnesium diets. These data suggested a relationship between alcohol and dietary magnesium. Vitale and his coworkers[11] recently reported that rats fed a diet containing cholesterol and cholic acid developed vascular sudanophilia which was reduced significantly by increased levels of dietary magnesium.

The present study investigated the interrelationships among these factors and the results showed that alcohol ingestion by rats increased vascular sudanophilia and serum cholesterol levels.

METHODS

Weanling albino male rats* weighing 50 to 55 grams were fed a basic diet consisting of purified casein (10 per cent), glucose (58.1 per cent), salt mixture (5.0 per cent),† celluflour (5.0 per cent), choline chloride (0.3 per cent), vitamin ADE mixture

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*Obtained from the Charles River Breeding Laboratories Boston, Mass.

†Salt mixture of Jones, J. H., and Foster, C.: J. Nutrition 24: 245, 1942, with the $CaCO_3$ and $MgSO_4$ removed.

TABLE I. THE EFFECT OF 20 PER CENT ALCOHOL ON SERUM CHOLESTEROL, HEART SCORE, KIDNEY PROFILE, AND GROWTH OF RATS FED A DIET CONTAINING CHOLESTEROL (1 PER CENT) AND CHOLIC ACID (0.3 PER CENT) (EXPERIMENT I)

DIETARY MAGNESIUM (MG. PER CENT)	ALCOHOL	SERUM CHOLESTEROL (MG. PER CENT)	HEART SCORE	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE FOOD INTAKE PER DAY (GM.)	AVERAGE LIQUID INTAKE PER DAY (ML.)	AVERAGE CALORIES PER DAY
				KIDNEY* SCORE	KIDNEY WEIGHT (MG.)	K/B RATIO				
24	–	653†	6	1	584	0.52	57	10.9	13.8	52.3
	+	965	7	0	511	0.56	46	7.9	6.8	45.4
96	–	660	5	1	496	0.44	58	9.8	12.0	47.0
	+	898	9	0	457	0.59	24	6.5	6.1	37.9
192	–	619	4	0	450	0.42	47	8.7	10.6	41.8
	+	1,086	3	0	394	0.48	35	6.8	6.1	39.3

*Renal tubular microlithiasis.
†Values represent the mean of 6 animals in each group.

(0.1 per cent),* $CaCO_3$ (1.5 per cent), and fat (Spry) (20 per cent). This diet was made atherogenic by the addition of 1.0 per cent of cholesterol and 0.3 per cent cholic acid with a proportionate reduction in the amount of glucose necessary to keep constant the percentage of protein and fat in the solid diet. The animals were housed in individual cages and fed ad libitum. Groups of animals were fed either the basic diet or the diet containing cholesterol and cholic acid. These two groups were further subdivided into those receiving either water or 20 per cent alcohol as the only source of fluid. Magnesium as magnesium oxide was added in concentrations varying from 6 to 192 mg. per 100 grams of diet.

Food, alcohol, and water intakes were measured daily. Animals were weighed biweekly, and observed for signs of magnesium deficiency, e.g., hyperexcitability and hyperemia of the ears, feet, and tail.

Animals were sacrificed at the end of 28 or 29 days. Serum cholesterol levels were determined according to the method of Carpenter and associates.[12] Sections of the gastrointestinal tract, kidney, liver, and heart were fixed in 10 per cent formalin for microscopic examination. The left kidney was decapsulated and weighed. Hearts were removed with the aorta attached and opened in a manner to expose the aortic and mitral valves. After fixation in formalin these specimens were stained with Sudan IV. The aortic and mitral valves, the intervalvular endocardium, and the aorta were examined by use of a dissecting microscope. The sudanophilia was estimated on a 0 to 4 plus scale by two of the authors working independently and the total vascular sudanophilia was designated as "heart score."[11, 13] The degree of calcium deposition in the renal tubules was evaluated in the manner previously described and designated as "kidney score."[13]

RESULTS

Table I shows that the serum cholesterol levels of animals drinking 20 per cent alcohol were significantly higher than those of animals fed only water. There was no apparent correlation between the level of dietary magnesium and serum cholesterol in animals fed alcohol or water.

Heart scores were 7, 9, and 3 for the groups fed 20 per cent alcohol and the 24, 96, and 192 mg. per cent magnesium diets, respectively. The daily average food intake for these 3 groups was approximately 7.0 Gm. The heart scores of animals fed water were 6, 5, and 4, and they ingested an average of 9.8 Gm. per day over the same period.

As was expected, high levels of dietary magnesium reduced the heart score of rats fed only water from 6 at 24 mg. per cent to 4 at 192 mg. per cent. Although those animals which were fed alcohol had significantly higher serum levels of cholesterol, the amount of vascular sudanophilia seen was significantly increased in only one instance. The group fed a diet containing 96 mg. per cent of magnesium and alcohol had a heart score of 9 while those fed the same level of dietary magnesium with water had a heart score of only 5.

The addition of alcohol resulted in decreased food intake as well as decreased total caloric intake.† Similarly, the decreased caloric intake was accompanied by a decreased rate of weight gain. The level of dietary magnesium was without effect on weight gain in both the alcohol and water-fed groups.

The kidney "profile" was evaluated by (1) gross appearance, (2) the weight of the kidney, (3) the ratio of kidney weight to body weight (K/B ratio), and

*A mixture of vitamins A, D, and E was prepared by adding 5 Gm. of halibut oil and 1 Gm. of alpha-tocopherol acetate to 44 Gm. of corn oil (Mazola). The following vitamins were added per kilogram of diet: 4 mg. thiamine hydrochloride, 8 mg. riboflavin, 4 mg. of pyridoxine hydrochloride, 25 mg. of calcium pantothenate, and 40 mg. of niacin.

†The caloric value of alcohol was calculated as 7 calories per gram of absolute alcohol.

TABLE II. THE EFFECT OF 20 PER CENT ALCOHOL ON SERUM CHOLESTEROL, HEART SCORE, KIDNEY PROFILE, AND GROWTH OF RATS FED A DIET CONTAINING CHOLESTEROL (1 PER CENT) AND CHOLIC ACID (0.3 PER CENT) (EXPERIMENT II)

DIETARY MAGNESIUM (MG. PER CENT)	ALCOHOL	SERUM CHOLESTEROL (MG. PER CENT)	HEART SCORE	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE FOOD INTAKE PER DAY (GM.)	AVERAGE LIQUID INTAKE PER DAY (ML.)	AVERAGE CALORIES PER DAY
				KIDNEY SCORE	KIDNEY WEIGHT (MG.)	K/B RATIO				
6	−(8)*	762	5.3	3	572	0.69	23	7.4	11.3	35.5
12	−(8)	645	5.6	3	624	0.61	41	9.2	12.7	44.2
	+(5)	784	6.9	2	440	0.56	19	6.1	6.2	36.1
24	−(8)	601	3.2	1	463	0.45	44	10.1	11.1	48.5
	+(6)	1,395	4.4	1	401	0.50	21	6.2	6.2	36.6
96	−(8)	597	1.9	1	462	0.45	42	9.6	10.4	46.1
	−(8)	1,145	4.0	0	408	0.52	18	6.3	5.9	36.7
192	−(8)	653	2.1	0	447	0.46	38	9.3	10.1	44.6
	+(7)	1,331	2.5	0	373	0.57	7	5.8	6.0	34.4
Correlation coefficient	−		−0.45 ($P<0.01$)			−0.47 ($P<0.01$)				
	+		−0.61 ($P<0.01$)			+0.15				

*Number of animals in each group.

TABLE III. THE EFFECT OF 20 PER CENT ALCOHOL ON SERUM CHOLESTEROL, KIDNEY PROFILE, AND GROWTH OF RATS FED A DIET FREE OF CHOLESTEROL AND CHOLIC ACID (EXPERIMENT II)

DIETARY MAGNESIUM (MG. PER CENT)	ALCOHOL	SERUM CHOLESTEROL (MG. PER CENT)	KIDNEY PROFILE			WEIGHT GAIN (GM.)	AVERAGE FOOD INTAKE PER DAY (GM.)	AVERAGE LIQUID INTAKE PER DAY (GM.)	AVERAGE CALORIES PER DAY (ML.)
			KIDNEY SCORE	KIDNEY WEIGHT (MG.)	K/B RATIO				
6	− (8)*	82	3	622	0.62	41	9.4	11.9	45.1
12	− (8)	90	3	679	0.59	54	11.5	12.2	55.2
	+ (4)	107	3	467	0.62	15	6.2	5.6	36.0
24	− (8)	88	1	490	0.45	50	9.9	11.8	47.5
	+ (6)	115	1	425	0.53	20	5.6	6.1	33.6
96	− (8)	76	0	476	0.40	58	10.1	11.9	48.5
	+ (7)	104	0	468	0.53	29	7.6	8.4	45.7
192	− (8)	75	0	482	0.40	59	10.5	11.1	50.4
	+ (7)	92	0	442	0.47	34	6.4	7.3	38.7
Correlation coefficient	−				−0.57 ($P<0.01$)				
	+				−0.79 ($P<0.01$)				

*Number of animals in each group.

(4) the kidney score. The effect of magnesium deficiency upon the kidneys was similar to changes previously described.[12-15] On gross examination kidneys showing the "magnesium effect" were usually large and pale, and the severe cases revealed surface speckling subsequently identified as tubular dilatation. Microscopic examination revealed the deposition of laminated calcium stones within the lumina of the collecting tubules of the outer third of the zona intermedia. We have called this change renal tubular microlithiasis. Occasionally, the microliths were found in the medullary tips, and their presence was not correlated with the severity of the deposits in the remainder of the medulla. Other microscopic findings included dilatation of the tubules surrounding the microliths with flattening of the cuboidial epithelium, focal epithelial hyperplasia, and local giant cell formation, as well as slight to moderate dilatation of the proximal nephron. Focal interstitial infiltrates of lymphocytes and plasma cells were also noted in the cortex and medulla.

Alcohol ingestion completely protected against renal tubular microlithiasis in this experiment (Table I). The kidney score of animals fed only water was 1 at 24 mg. per cent and was decreased to a score of 0 at the highest level of dietary magnesium. The average weight of the kidneys of the alcohol-fed animals was slightly less than that of animals on water. The K/B ratio was reduced from 0.52 at 24 mg. per cent to 0.42 at 192 mg. per cent in animals fed water. However, the K/B ratio was always higher in alcohol-fed animals.

In Experiment II (Table II) two lower levels of dietary magnesium were added. Animals fed 6 mg. per cent of magnesium were not given alcohol since in our experience these animals have not survived such a regimen. Animals fed alcohol instead of water had significantly higher levels of serum cholesterol despite the fact that they ingested approximately 35 per cent less food. The average serum cholesterol level for animals ingesting water varied only from 596 to 762 mg. per cent regardless of the level of dietary magnesium. Alcohol-fed animals had serum cholesterol levels ranging from 800 to approximately 1,400 mg. per cent.

The heart scores of animals fed only water were 5.3 at 6 mg. per cent magnesium, 5.6 at 12 mg. per cent, 3.2 at 24 mg. per cent, and approximately 2 at 96 and 192 mg. per cent. Heart scores of animals fed alcohol, however, were 6.9 at 12 mg. per cent magnesium, 4.4 at 24 mg. per cent, 4.0 at 96 mg. per cent, and 2.1 at 192 mg. per cent. The correlation coefficient calculated for dietary magnesium versus heart score was 0.45 for the group fed water and 0.61 for the group fed alcohol, both of which are significant at the 0.01 level ($P<0.01$).

When cholesterol and cholic acid were omitted from the diet (Table III) the average serum cholesterol level (103 mg. per cent) of the alcohol-fed group was slightly higher than that of the water-fed animals (82 mg. per cent). Dietary magnesium had no effect on serum cholesterol levels in either group. The heart scores, as expected, in this group were zero.

Maximal weight gain of alcohol-fed animals occurred at dietary magnesium levels of 96 to 192 mg. per cent while water-fed animals grew maximally at 12 mg. per cent.

TABLE IV. EFFECT OF 20 PER CENT ALCOHOL AND MAGNESIUM ON GASTROINTESTINAL HEMORRHAGE

MAGNESIUM (MG. PER CENT)	ATHEROGENIC DIET	NONATHEROGENIC DIET
12	3/8*	4/8
24	2/8	2/8
96	0/8	1/8
192	1/8	1/8

*Number of animals dying with GI hemorrhage.

In contrast to Experiment I, alcohol did not prevent renal tubular microlithiasis. The addition of magnesium to the nonatherogenic diet with and without alcohol and to the atherogenic diet without alcohol significantly reduced the K/B ratio but did not reduce the elevated ratio in animals fed the atherogenic diet and alcohol (Tables I, II, and III). In general, the atherogenic diet alone caused greater K/B ratios than did the nonatherogenic diet. However, even when large amounts of magnesium were added to the atherogenic diet the K/B ratio remained elevated. It was also noted that alcohol tended to increase the K/B ratios of animals fed the atherogenic and nonatherogenic diets.

Fourteen alcohol-fed rats in Experiment II died with moderate to marked gastrointestinal hemorrhage. The largest number of deaths occurred in those rats fed the low-magnesium diets (Table IV). There was no significant difference between the groups fed either the atherogenic or nonatherogenic diet. The stomachs and intestines were partially to completely filled with changed black or brown blood. On gross examination, a few stomachs were focally congested and showed petechial-like hemorrhages in the glandular portion. No ulcerations were found in either stomach or duodenum. Microscopic examination of the stomach and duodenum revealed only slight to moderate autolysis of the glandular portion of the mucosa.

DISCUSSION

Weanling rats fed the cholesterol-cholate diets regularly developed hypercholesterolemia. The addition of 20 per cent alcohol to this diet caused as much as a twofold increase in the degree of hypercholesterolemia, although the food intake was approximately one-third less. This is essentially in agreement with the studies of Eberhard[6] who noted elevated serum cholesterol levels in alcohol-fed rabbits on an atherogenic diet.

The cause of the elevated serum cholesterol levels associated with alcohol intake is not clear. Whether alcohol facilitates the absorption of cholesterol, or promotes endogenous synthesis of cholesterol, or impedes the degradation of endogenously produced cholesterol are questions which remain to be investigated.

On gross examination, livers of both the alcohol and water-fed animals on the atherogenic diet were enlarged and pale. Microscopic examination revealed slight fatty vacuolization of hepatic cells in both groups of animals. Thus, it was not possible to correlate the variations in serum cholesterol levels with the liver morphology.

Animals ingesting alcohol did not consume a 10 per cent protein diet. Since the amount of alcohol ingested comprised part of the total caloric intake, the

adjusted value of the protein in these diets was approximately 8 per cent. This decreased protein intake could account for part of the rise of serum cholesterol observed in the alcohol-fed animals. Fillios and associates[16] demonstrated that a reduction in the amount of protein from 10 per cent to 5 per cent in atherogenic diets resulted in an increase of serum cholesterol levels of approximately 30 per cent. However, the increases in cholesterolemia associated with alcoholic ingestion (up to 100 per cent) are too great to be accounted for by a 2 per cent reduction in protein intake.

The possibility was considered that alcohol ingestion might have resulted in a relative choline deficiency superimposed upon a magnesium deficiency producing higher heart scores. Hartroft and co-workers[17] reported early atheroma-like lesions in the coronary arteries, carotid arteries, and aortas of rats fed a hypolipotropic diet; and Wilgram and Hartroft[18] stated that young male rats fed an atherogenic hypolipotropic diet developed coronary lipoidosis and aortic sclerosis. However, Wilgram and his co-workers[19] later reported that dietary choline was necessary for the production of atheroma-like lesions and recent studies[20] have shown that atheromatous plaque formation does not take place readily in the hearts and aortas of choline-deficient rats fed an atherogenic diet. Furthermore, Weiss and associates[21] demonstrated that the plasma cholesterol level in choline-deficient animals was reduced and that the addition of lipotropic substances to an atherogenic diet significantly raised the cholesterol level. Similarly, Mann and co-workers[22] reported that choline was essential for the production of sustained hypercholesterolemia in *Cebus* monkeys fed diets high in cholesterol. Thus, in view of the elevated serum cholesterol levels and heart scores in the alcohol-fed groups it was concluded that the dietary choline was adequate to satisfy the choline requirement of animals in this study.

As seen in Tables I, II, and III the fluid intake of the alcohol-fed animals was greatly reduced. It is improbable that dehydration caused part of the increase in serum cholesterol levels, since the results of preliminary studies in this laboratory showed no differences between the hematocrits of water and alcohol-fed rats.

In Experiment I the heart scores of alcohol-fed animals were essentially the same as, and in one group higher than, water-fed animals. In Experiment II, the heart scores of the alcohol group were consistently higher than those of the water-fed animals. As expected, high levels of magnesium tended to decrease the heart scores, but did not completely eliminate heart valve and aortic sudanophilia. There was no apparent effect of magnesium upon serum cholesterol levels. It would appear that hypercholesterolemia is essential for the production of sudanophilia but that it is not directly proportional to the level of cholesterolemia. These observations are in agreement with previous studies[11] in which a disassociation between serum cholesterol levels and the extent of sudanophilia was noted.

Since this study was performed on the weanling rat, the relevance of the findings with respect to human atherosclerosis is uncertain. The validity of using the cholesterol-fed rat as a model for the human disease remains to be

proved. Furthermore, the alleged reduction in atherosclerosis attributed to alcoholism may indeed be due to the effect of alcohol upon the dietary habits of alcoholics, who in general, eat fewer calories, fat, and protein during periods of intensive drinking. Further clinical studies correlating, at least, dietary habits, serum cholesterol, and magnesium levels with the degree of atherosclerosis are indicated before one can relate alcohol ingestion per se to atherogenesis.

The apparent contradiction between the results reported here and those of Eberhard[6] and more recently Nichols and associates[7] who found that alcohol did not promote the development of atherogenesis in rabbits and chickens may be partially explained by the higher levels of protein used by these investigators. It seems likely that the atherogenic effect of alcohol is mediated in part by an increased magnesium requirement. This effect is apparent only at low levels of protein. Vitale and co-workers[11] demonstrated that high dietary magnesium did not exhibit any protective effect on heart sudanophilia when 20 per cent protein was incorporated into diets containing 1 per cent cholesterol and 0.3 per cent cholate. Thus, a diet high in protein may mask the effect of alcohol in increasing the magnesium requirement.

The results of these studies and others[23] raised the question as to whether or not cholesterol and cholic acid aggravated the kidney lesions attributed to low-magnesium diets. It is not possible to distinguish the kidney of an animal fed an atherogenic diet from that of an animal fed a nonatherogenic diet by weight alone. In experiments reported by Hellerstein and associates[13] feeding cholesterol and cholic acid usually increased the amount of renal tubular microlithiasis and, indeed, tubular calcification has been used as an indication of magnesium deficiency. In the present study, this finding was not constant. On the other hand, the ratio of kidney weight to body weight was always elevated in animals fed cholesterol and cholic acid, and it appeared that the gross morphology, coupled with the kidney to body weight ratios, was a more consistent index of magnesium deficiency in cholesterol and cholic acid-fed animals than was renal tubular calcification. In subsequent studies, Vitale and associates[23] also made these observations.

Gastrointestinal hemorrhage was noted in 14 rats which died prior to the termination of Experiment II. All 14 animals were on 20 per cent alcohol and there was no significant difference between the groups fed atherogenic and nonatherogenic diets. Increased levels of dietary magnesium protected the rat against gastrointestinal hemorrhage. Unfortunately, postmortem autolysis interfered with the histologic study of the upper gastrointestinal tract and may have masked gastric lesions.

SUMMARY

Weanling male rats were fed diets made atherogenic by the addition of 1 per cent cholesterol and 0.3 per cent cholic acid. One group of animals received 20 per cent alcohol as the only source of fluid and another group received water. Magnesium was added in the form of magnesium oxide in varying concentrations from 6 to 192 mg. per 100 Gm. of diet.

The results of this study demonstrated that rats fed an atherogenic diet and 20 per cent alcohol ate approximately 35 per cent less food but had higher

serum cholesterol levels and more extensive vascular sudanophilia than rats fed an atherogenic diet with water. High levels of dietary magnesium lowered the amount of lipid deposition.

It was also shown by the kidney profile and weight gain that alcohol increased the magnesium requirements of animals fed the basic diet, and that the addition of cholesterol and cholic acid accentuated the deficiency. Possible mechanisms for these observations were discussed.

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SERUM CHOLESTEROL LEVELS IN ATHEROSCLEROTIC PATIENTS TREATED WITH ORAL ANTICOAGULANTS

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ANTICOAGULANT therapy has proved to be of value in the treatment of the thrombotic complications of coronary atherosclerosis. However, other possible actions of the oral anticoagulants have not been intensively studied. The direct-acting anticoagulant, heparin, has antilipemic properties and prolonged heparin treatment of patients with myocardial infarction has been claimed by Engelberg[1] to result in a lowered mortality and morbidity in such patients. This may be associated with the antilipemic properties of heparin.

Although the oral anticoagulants have a different mechanism of action than heparin and need not necessarily have actions similar to heparin on serum lipids, nevertheless it seemed of interest to study the effect of these agents on serum lipids of subjects who had been placed on these drugs because of thrombotic episodes or evidence of impending thrombosis.

METHODS

Five groups of patients were studied. The first group consisted of 8 individuals treated with phenylindanedione; the second consisted of 10 subjects who received acenocoumarin; and a third group was composed of 6 patients who were given ethyl biscoumacetate.* The age range in each of these 3 groups was from 50 to 70 years. All these patients had evidence of impending myocardial infarction or had experienced a recent myocardial infarction; these two types were distributed equally throughout the three groups.

Since many of the atherosclerotic subjects in all groups had shown evidence of vascular disease, the majority had been placed on diets of moderate fat and caloric intake months or weeks previous to the study. The rigidity of adherence to these diets is not known, but the diets of all patients, as far as could be ascertained, remained the same during the periods of observation.

The anticoagulants were administered orally in divided doses daily in amounts sufficient to maintain the prothrombin time, estimated by the one-stage method of Quick, between 25 and 30 per cent of normal. Blood samples were taken at intervals over the first 10 weeks of therapy and again over a period between 24 and 32 weeks after starting therapy.

Another group, consisting of 7 middle-aged atherosclerotic subjects, receiving no anticoagulant therapy, were observed over a 6-week period. A fifth group of 5 atherosclerotic individuals were treated with heparin in doses insufficient to produce anticoagulation.

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*Acenocoumarin is marketed as Sintrom, ethyl biscoumacetate as Tromexan. Both preparations were supplied by Geigy Pharmaceuticals, Montreal.

In the latter group all subjects had blood samples taken before starting treatment. They were then given 200 mg. of herapin subcutaneously every 48 hours over a 6-week period; blood samples were taken 2 days after the first dose of heparin and thereafter at approximately weekly intervals 48 hours after an injection of heparin and just prior to the next injection.

The blood samples were centrifuged and the separated serum either analyzed the same day or quickly frozen and stored at -15° C. and analyzed later. The following determinations were made on the sera of the patients: total serum cholesterol by the method of Pearson and associates[2]; total serum lipids using the turbidimetric method of Kunkel and co-workers[3]; and serum lipoprotein was determined by paper electrophoresis after the method of Jencks and associates.[4] The separated lipoproteins were stained with oil red O. The stained lipoproteins were eluted with methanolic chloroform and the optical density measured in a Beckman model B spectrophotometer. The β-lipoprotein was expressed as a per cent of the sum of the optical densities of the α plus β lipoprotein lipid. Duplicate determinations agreed within 7 per cent. Lipoprotein determinations made on the 24- to 32-week samples were measured with an automatic scanning device (Spinco Analytrol). Results obtained by scanning and eluting the same strips were similar.

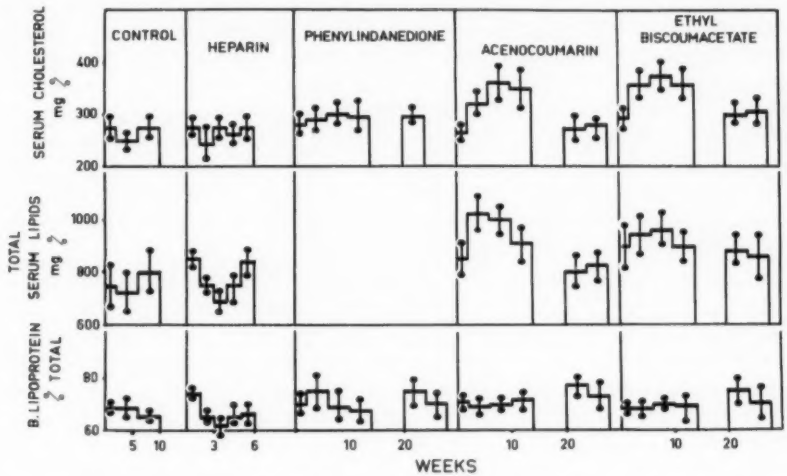

Fig. 1.—The serum cholesterol, total serum lipids, and lipoprotein levels in atherosclerotic patients receiving anticoagulants.

RESULTS

It will be seen from Fig. 1 that significant ($p < 0.05$) elevations occurred following the initiation of treatment with the coumarin derivatives, acenocouramin and ethyl biscoumacetate. However, when investigated again after 24 to 32 weeks, the serum cholesterol in these patients had returned to the levels found at the beginning of anticoagulant therapy. This rise following treatment was not apparent in the similar patients who received phenylindanedione. The control group of untreated atherosclerotic patients showed no significant variation in the serum cholesterol over a 10-week period of study. In the heparin-treated group no significant alterations in cholesterol were found over the 6-week period this therapy was carried on.

The total serum lipids tended to parallel the serum cholesterol levels; significant elevations were noted following acenocoumarin and ethyl biscoumacetate therapy, but no real changes occurred in the untreated group. In the heparin-treated group the total serum lipids fell significantly from the pretreatment levels but this decrease was not maintained.

No significant changes were observed to occur in the β-lipoprotein measured as a per cent of the total lipoprotein except in the subjects treated with heparin. In this group there was an early fall in the proportion of β-lipoprotein which was maintained for 2 weeks and thereafter tended to return to pretreatment values in some of the subjects.

DISCUSSION

There is an apparent difference between the serum cholesterol of the groups treated with acenocoumarin and ethyl biscoumacetate and the group treated with phenylindanedione. In the acenocoumarin and ethyl biscoumacetate groups, significant elevations in serum cholesterol occurred but these did not appear to be maintained. No changes were apparent in similar patients treated with phenylindanedione. Bernard-Griffiths[5] has reported that a combination of small doses of Dicumarol and choline given to atherosclerotic individuals produced a slight increase in serum cholesterol. Although not strictly comparable with our studies, this report and our findings with the coumarin derivatives are in accord. Reiniš and co-workers[6] have reported that administration of ethyl biscoumacetate to cholesterol-fed rabbits accelerates the rate of increase in serum cholesterol and rate of evolution of atheromatous lesions which usually follow cholesterol feeding in the rabbit.

There are several possible explanations for the difference in the serum cholesterol values of the patients treated with the coumarin derivatives. The coumarins may have brought about a decreased catabolism or an increased synthesis or absorption of cholesterol, or they may have caused a mobilization of cholesterol from vascular plaques. Whatever the mechanism, it does not appear to be persistent, for by the twentieth week of treatment the cholesterol level was back to pretreatment values.

The lack of response of serum cholesterol in the heparin-treated group is in accord with the findings of others.[7, 8] However, the total serum lipids and the β-lipoprotein fell in these individuals. These results are in agreement with those of Soffer[7] who reported a fall in total serum lipids in subjects treated with heparin. These changes may be in some way related to the clearing of an alimentary lipemia which follows heparin injection. In the present series, all subjects showed initial changes in total serum lipids and lipoproteins after heparin injections were started. However, in 3 subjects these changes were not maintained and the lipid levels and β-lipoprotein were observed to have returned to pretreatment levels in the blood samples taken 4 weeks after the institution of treatment.

The findings reported here indicate that dicoumarin derivatives, particularly acenocoumarin, temporarily elevate the serum cholesterol and total serum lipids.

Whether or not this is of any importance in the use of those agents as anticoagulants in atherosclerotic patients remains to be determined.

SUMMARY

Atherosclerotic subjects were treated with heparin or the oral anticoagulants, phenylindanedione, or dicoumarin derivatives (acenocoumarin and ethyl biscoumacetate), and the serum cholesterol, total serum lipids, and β-lipoproteins were measured.

In the phenylindanedione-treated group no significant change occurred in any of these serum lipids. In patients treated with acenocoumarin and ethyl biscoumacetate, elevations occurred in the serum cholesterol and total serum lipids which were maintained for at least 10 weeks but were back to pretreatment levels after 24 to 32 weeks of therapy.

In none of the groups treated with oral anticoagulants were any significant alterations in the β-lipoproteins noted.

In the group treated with heparin no change was noted in the serum cholesterol levels but significant falls were found in the levels of total serum lipids and beta lipoproteins.

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STUDIES ON ERYTHROPOIESIS

X. The Use of Bone Marrow Tissue Culture in Demonstrating Erythropoietin

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IN RECENT years, the kinetics and controlling mechanisms of erythrocyte production have been studied extensively. One of the most important and interesting results of these investigations is the demonstration that a humoral substance(s) (erythropoietin) is responsible, at least in part, for the increased erythropoiesis in response to hemorrhage,[1-4] hemolytic anemia,[5-6] hypoxia,[7-8] and cobalt administration.[9] Furthermore, indirect evidence has been accumulated showing that the amount of this factor(s) is decreased when the production of erythrocytes is reduced as in transfusion-induced polycythemia[10] and hyperoxia.[11]

To date, nearly all assays of erythropoietic activity have been carried out in whole animals. Increases in reticulocyte, hemoglobin, and hematocrit values,[2-7] as well as the increase in radioiron uptake,[1, 8] have been used as parameters in assessing the effect of erythropoietin-rich serum on normal, starved hypophysectomized, or polycythemic animals. Qualitative estimates of erythropoietic activity in the spleen and bone marrow have also been used as evidence of the presence or absence of erythropoietin.[10] However, only one report[12] of the quantitative estimation of the effect of serum rich in erythropoietin on proliferation of erythrocyte precursors was found in the literature.

The improvement in tissue culture techniques has made it possible to maintain tissue cells in vitro. It is the purpose of this paper to present some experiments wherein the effect of different sera upon bone marrow cells grown in tissue culture is measured quantitatively.

MATERIALS AND METHODS

The chambers used in these experiments for the cultivation of the marrow cells were a modified version of the Mackaness and Smith monocyte culture chamber† (Fig. 1, *A* and *B*).[13] This consists of a block of unplasticized Perspex 4.9 by 3.2 by 0.22 cm. In the center, circular holes with a diameter of 23 mm. are counterbored from each side to within 1.5 mm. of each other, and a hole, 19 mm. in diameter, is drilled completely through. Thus the central hole is surrounded by a recessed border on both sides. From the side, two small holes, 0.7 mm. in diameter, are drilled into the central hole. The plug consists of two parallel

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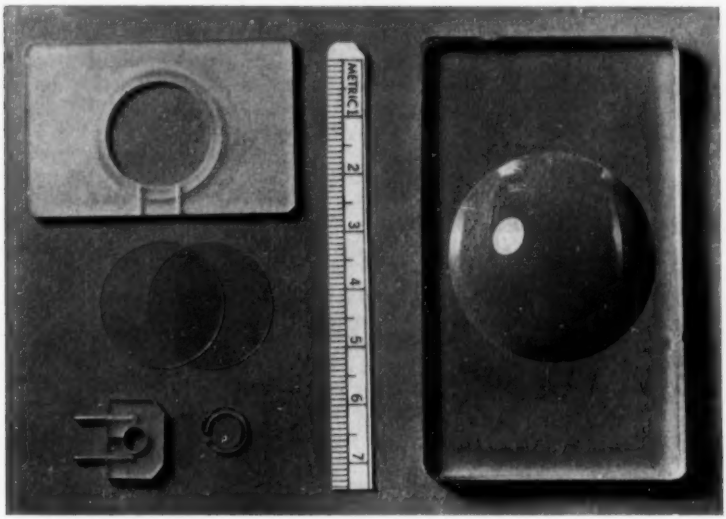
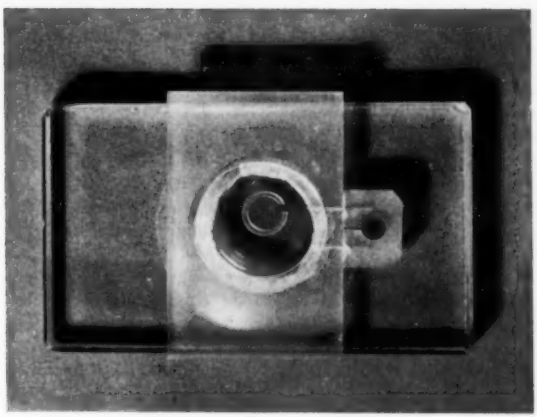
†Obtained from H. G. East & Company, Oxford, England, and constructed in the Engineering Division of the Argonne Cancer Research Hospital, Chicago, Ill.

wires of slightly smaller diameter set in a small piece of plastic at the same distance apart as the two lateral holes. A Perspex ring, 1 mm. high and 6 mm. inside and 8 mm. outside diameters, from which a small arc has been removed completes the apparatus.

A.





B.

Fig. 1.—*A*, Component parts of the modified Mackaness and Smith monocyte culture chamber before assembly. Upper left, main block; middle left, cover glasses; lower left, plug and internal ring; right, inverted shallow-well glass slide used to support main block during sterilization. *B*, Modified Mackaness and Smith monocyte culture chamber fully assembled.

The plastic chamber is constructed after sterilization in dry heat (100 to 105° C.) for 1 hour, using paraffin (melting point 56 to 58° C.). A chemically clean and sterile circular cover slip, 22 mm. in diameter, is placed on one recessed border and secured with melted wax. The chamber is inverted after cooling, and the previously waxed central ring is placed

so that a needle may be inserted through a lateral hole and into the center of the ring through the missing arc. A cover glass is placed on the top recessed edge, and the bottom cover glass is gently heated to melt the wax on the central ring, thus securing it. The top glass is then secured with melted wax.

The culture medium used was Earle's salt solution* with added vitamins,* amino acids,* and glutamine* in amounts twice Eagle's specifications.[14-16] The serum used was taken under sterile conditions by cardiac puncture from Holtzman strain rats that had been fasted overnight. The following sera were tested: (1) serum from normal rats weighing 200 to 300 grams (normal serum); (2) serum from rats that had been made anemic by hemorrhage on two consecutive previous days and in which the hematocrit was less than 25 per cent (anemic serum); (3) serum from rats given cobaltous chloride (25 μM per 100 Gm. body wt.) with tracer amounts of radioactive cobalt^{60} ten hours previously (cobalt serum); (4) normal rat serum with an amount of cobaltous chloride added in vitro equal to the amount present in the cobalt serum (cobalt control serum); and (5) serum from rats that had been rendered polycythemic by tranfusion of erythrocytes equal to 4 per cent of the body weight given over a period of 3 days and in which the hematocrit was 65 to 70 per cent (polycythemic serum).

The marrow cells were obtained by means of sterile dissection of both femurs of a rat killed by cervical fracture and were placed in 5 ml. of Eagle's salt solution to which Versenate (disodium ethylenediaminetetraacetate) had been added in a concentration of 1.5 mg. per milliliter. The cells were agitated, and the multicellular clumps allowed to settle to the bottom by tilting the tube at an angle of 45 degrees in ice water for 5 to 10 minutes. The supernatant suspension of single cells and smaller clumps of cells was removed and centrifuged for 10 minutes at 1,200 r.p.m.; the acellular supernatant was removed. The cells were resuspended in enough Eagle's medium (without serum) to make a concentration of 6,000 to 8,000 nucleated cells per cubic millimeter. Two parts of this cellular suspension were added to 1 part of each of the sera being tested. Likewise, 2 parts of Eagle's medium were added to 1 part of each of the sera, making the medium complete.

Inside the ring of each chamber was placed 0.06 ml. of the cellular suspension by means of a tuberculine syringe equipped with a No. 22 needle. The space between the ring and the main part of the chamber was partially filled with the corresponding complete medium, leaving an air bubble. The plug was dipped in a 1:1 mixture of paraffin and yellow petrolatum, and the wires were inserted into the 2 lateral holes. The chambers were incubated at 37° C. and rotated at intervals of 6 to 12 hours. Ten to fifteen replicate cultures were prepared for each serum tested.

At specified time intervals, the upper cover glass was removed and the ring loosened. The medium was agitated gently to remove all cells from the bottom and was carefully aspirated and measured with a tuberculin syringe graduated in 0.01 ml. units. The chamber was washed twice with 0.2 ml. of 2 per cent acetic acid, and the recovered wash fluid was measured. No cells remained on the bottom cover glass. The number of nucleated cells per cubic millimeter was counted without further dilution on a regular Neubauer hemocytometer; two separate aliquots were used. The total number of cells removed was calculated. This process was performed on 3 chambers of a particular serum at each time.

Slides for differential count were made on 2 chambers of each serum at each time. The top cover glass was detached, and the supernatant fluid was removed carefully while the central ring was still attached. The cells remaining on the bottom cover glass were quickly air-dried and stained with Wright's stain after removing the cover glass from the chamber and the ring from the cover glass. The cover glasses were mounted, cells downward, on regular glass slides and labeled so that they could be counted as unknowns. Differential count of 1,000 cells on each slide was performed in a systematic fashion, counting the cells in separate microscope fields along the horizontal, vertical, and (occasionally) diagonal diameters of the cellular area.

*Obtained from Microbiological Supplies, Bethesda, Md.

From these data, the total number of any particular cell type or types can be calculated.

$$\text{Number of type A cells} = (\text{total cells per mm.}^3 \times \text{mm.}^3) \times \frac{\%\text{ of type A cells}}{100}.$$

RESULTS

The number of erythrocytic precursors was plotted against time of incubation (see Figs. 2, 3, and 4). In the presence of normal serum the number of these cells decreased linearly throughout the 24-hour period. In cultures incubated with anemic serum, the number of these cells increased during the first 12 hours and decreased during the second 12 hours. Cobalt serum elicited the same response, and cultures incubated in cobalt control serum behaved similar to those in normal serum. With polycythemic serum, the number of erythrocyte precursors at 12 hours was significantly below the number in cultures using normal serum. During the following 24 hours (12 to 36 hour incubation) the number of erythrocyte precursors in the polycythemic cultures was lower than in the normal cultures but the difference was not so striking.

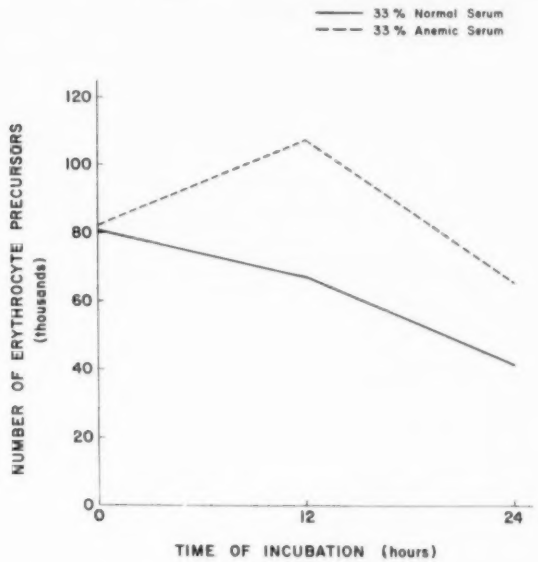


Fig. 2.—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal and anemic sera.

The number of each of the erythrocyte precursors (rubriblast, prorubricyte, rubricyte, and metarubricyte) was analyzed for each of the time intervals (Tables I, II, and III). In 3 out of the 4 experiments (anemic or cobalt serum), it was observed that at 12 hours the number of metarubricytes was increased above the original number at 0 hour; this increase was maintained

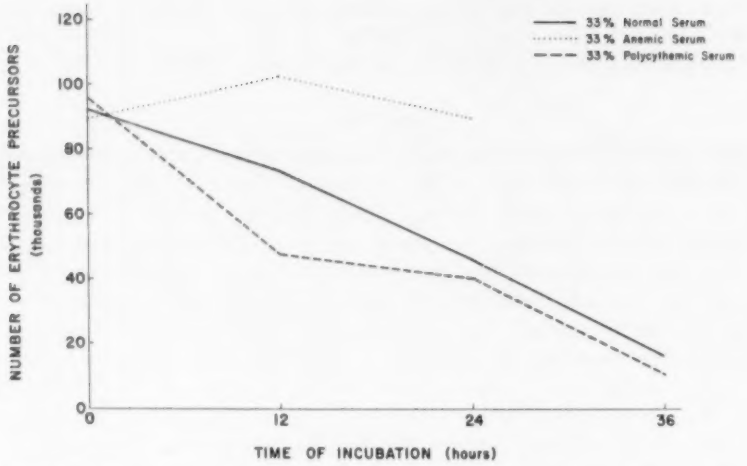


Fig. 3.—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal, anemic, and polycythemic sera.

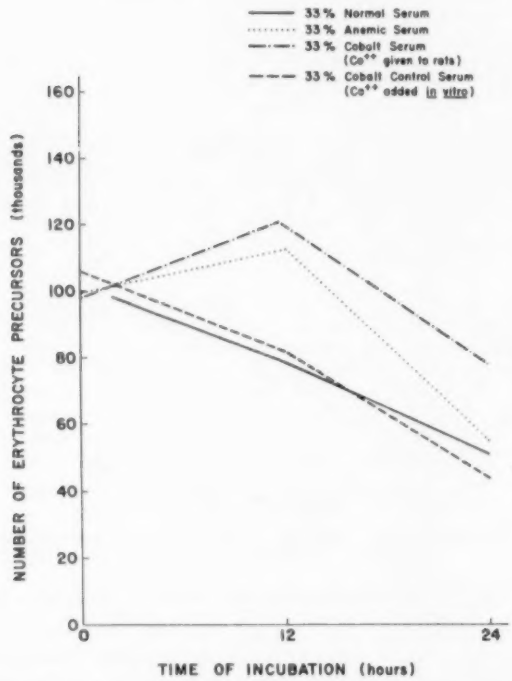


Fig. 4.—Total number of erythrocyte precursors in bone marrow tissue cultures grown in normal, anemic, cobalt, and cobalt control sera.

through 24 hours. In one experiment with anemic serum (Experiment 3), the number of metarubricytes was only slightly higher at 12 hours and had decreased by 24 hours. At this same interval the number of rubricytes and prorubricytes was increased over the original number and was considerably higher than the number of these cells in normal cultures. During the second 12-hour period, the number of these cells decreased at a rate greater than that seen in normal cultures. Rubriblasts are so scarce in these cultures that changes in their number cannot be accurately assessed.

TABLE I. (EXPERIMENT 1) NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED WITH NORMAL AND ANEMIC SERA AFTER VARYING INTERVALS OF INCUBATION

PRECURSOR	0 HOUR	12 HOURS		24 HOURS	
		NORMAL	ANEMIC	NORMAL	ANEMIC
Total cell count	301,240	267,430	271,170	199,630	245,480
Metarubricyte					
Number	26,810	44,130	45,010	30,540	43,690
Per cent	8.9	16.5	16.6	15.3	17.8
Rubricyte					
Number	28,610	17,380	37,690	8,980	15,710
Per cent	9.5	6.5	13.9	4.5	6.4
Prorubricyte					
Number	24,010	5,350	21,960	1,200	5,150
Per cent	8.0	2.0	8.1	0.6	2.1
Rubriblast					
Number	2,410	2,670	2,980	200	490
Per cent	0.8	0.1	1.1	0.1	0.2
Total erythrocyte precursors					
Number	79,430	69,530	107,640	40,920	65,040
Per cent	27.2	25.1	39.7	20.5	26.5

TABLE II. (EXPERIMENT 2) NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED IN NORMAL, ANEMIC, AND POLYCYTHEMIC SERA AFTER VARYING INTERVALS OF INCUBATION

PRECURSOR	12 HOURS			24 HOURS			36 HOURS	
	NORMAL	ANEMIC	POLYCYTHEMIC	NORMAL	ANEMIC	POLYCYTHEMIC	NORMAL	POLYCYTHEMIC
Total cell count	252,380	260,050	228,320	208,310	252,680	187,160	143,290	135,100
Metarubricytes								
Number	40,380	64,490	31,050	31,250	65,410	26,760	10,750	6,620
Per cent	16.0	24.8	13.6	15.0	25.9	14.3	7.5	4.9
Rubricytes								
Number	22,460	26,780	9,580	10,830	17,180	10,290	4,300	2,560
Per cent	8.9	10.3	4.2	5.2	6.8	5.5	3.0	1.9
Prorubricytes								
Number	9,340	8,580	7,300	4,170	5,550	2,990	500	800
Per cent	3.7	3.3	3.2	2.0	2.2	1.6	0.4	0.6
Rubriblasts								
Number	500	2,080	200	0	700	200	200	100
Per cent	0.2	0.8	0.1	0.0	0.3	0.1	0.2	0.1
Total erythrocyte precursors								
Number	72,680	101,930	48,130	46,250	88,840	40,240	15,750	10,080
Per cent	28.8	39.2	21.1	22.2	35.2	21.5	11.1	7.5

TABLE III. (EXPERIMENT 3) NUMBERS AND PERCENTAGES (OF TOTAL NUCLEATED CELLS) OF ERYTHROCYTE PRECURSORS IN BONE MARROW CULTURED IN NORMAL, ANEMIC, COBALT CONTROL, AND COBALT SERA AFTER VARYING INTERVALS OF INCUBATION

PRECURSOR	0 HOUR	12 HOURS				24 HOURS			
		NORMAL	ANEMIC	COBALT CONTROL	COBALT	NORMAL	ANEMIC	COBALT CONTROL	COBALT
Total cell count	232,990	245,420	217,230	249,420	246,560	184,620	202,030	155,790	229,790
Metarubricytes									
Number	58,010	49,080	59,030	55,120	67,800	38,400	33,130	35,050	69,170
Per cent	24.9	20.0	27.3	22.1	27.5	20.8	16.4	22.5	30.1
Rubricytes									
Number	27,260	21,840	33,020	21,700	25,400	9,800	11,920	7,480	10,340
Per cent	11.7	8.9	15.2	8.7	10.3	5.3	5.9	4.8	4.5
Prorubricytes									
Number	14,450	6,630	18,250	4,490	21,700	2,400	8,280	470	4,360
Per cent	6.2	2.7	8.4	1.8	8.8	1.3	4.1	0.3	1.9
Rubriblasts									
Number	1,630	1,000	2,390	200	6,160	500	1,000	0	0
Per cent	0.7	0.4	1.1	0.1	2.5	0.3	0.5	0.0	0.0
Total erythrocyte precursors									
Number	101,350	78,550	112,690	81,510	121,060	51,100	54,330	43,000	83,870
Per cent	43.5	32.0	52.0	32.7	49.1	27.7	26.9	27.6	36.5

When polycythemic serum was used there was a decrease in the number of metarubricytes and rubricytes that was more rapid than that seen in normal cultures during the first 12 hours.

DISCUSSION

Normal Serum.—The linear decrease of erythrocyte precursors in normal cultures is the product of interaction of several factors. The number of precursors present at any time represents the balance between proliferation, cell death, and maturation to anuclear erythrocytes during the preceding period of time. Cell death may be large in these cultures because the environmental conditions in the chamber are suboptimal when compared with marrow in situ. Proliferation is probably decreased and maturation increased by "progressive maturation of the culture," i.e., the tendency of all the cells in the culture to mature and thus become incapable of dividing and maintaining the line. Osgood[17] emphasized that when an immature cell divides, one of the daughter cells must remain immature and the other must mature if the normal numerical relationships are to be maintained. There is good evidence that this delicate balance is not maintained in these cultures since the proportion of metarubricytes and segmented neutrophils to total erythrocyte precursors and total granulocytes, respectively, increases and the proportion of more immature forms decreases with time. Thus all factors tend to decrease the number of erythrocyte precursors during any time interval.

Anemic Serum.—The increase in the total number of erythrocyte precursors during a 12-hour incubation with anemic serum suggests that the erythropoietic-stimulating factors(s) (erythropoietin) is present in anemic serum in a form that is directly usable by the bone marrow cells. The proliferative stimulatory effect of this factors(s) is apparently upon the prorubricytes and rubricytes.

As in the normal cultures, the decline in erythrocyte precursors during the second 12 hours is the result of 3 processes: proliferation, cell death, and maturation. Again, progressive maturation of the culture is occurring; this tends to offset any stimulatory effect of the serum on proliferation by reducing the number of cells that are capable of division. It is impossible to state with certainty whether maturation of single erythrocyte precursors and/or progressive maturation of the culture is hastened by the stimulatory effect of the anemic serum. The more rapid decline in rubricytes and prorubricytes during this period suggests that both individual and progressive maturation are probably hastened by the active serum.

Cobalt and Cobalt Control Serum.—When cobalt serum was used, there was evidence of stimulation to the same degree as in anemic serum. When cobalt control serum was used, no such activity was demonstrated. These facts substantiate the findings of Goldwasser and associates[9, 18] that the erythropoietic effect of cobalt is mediated through a humoral mechanism (erythropoietin). In addition, the almost exact correspondence of erythrocyte precursors in the cultures grown with cobalt control serum and those grown with normal serum

indicates that there is no direct effect of the ion on the bone marrow at these concentrations. These results are in agreement with the observations of Laforet and Thomas[19] who found no direct stimulatory effect of cobalt ion on bone marrow when oxygen levels and heme synthesis were analyzed in cultures.

Polycythemic Serum.—When animals are rendered polycythemic by transfusion, the amount of circulating erythropoietin is presumably reduced because the amount of oxygen at the site of production is increased and thus the stimulus for production is reduced.[11] Erythropoiesis in the transfusion-induced polycythemic mouse spleen is reduced.[10] In the present experiments, the lower number of erythrocyte precursors in polycythemic cultures as compared with that in normal cultures at 12 hours is presumptive evidence that the amount of erythropoietin is greater in normal serum than in polycythemic serum and that erythropoietin is necessary to maintain a "normal" state of erythropoiesis. (However, the presence of an inhibitor of erythropoiesis in the polycythemic serum cannot be ruled out with absolute certainty.) Erythropoietin appears to be one of the controlling mechanisms in the normal state and not merely an emergency mechanism to stimulate erythropoiesis during stress.

Errors of Measurement.—The over-all effects of the various treatments are clear from examination of Figs. 2 to 4. Although 3 chambers were used to determine each total cell count, ordinarily only 1 or 2 differential counts were made for each treatment at each time. Thus, we can measure directly the precision of a total cell count but not that of differential count or of their product, the total erythrocyte precursor count. However, in some preliminary experiments replicate differentials counts were made, and we have used these to measure the precision of differential counts.

It was found that the coefficient of variation (standard deviation divided by the mean count) of repeated total counts was approximately 12 per cent (somewhat smaller for the zero hour counts) and consequently the coefficient of variation of the average of 3 such counts, being less by a factor of $1/\sqrt{3}$, is approximately 7 per cent. For counts in the neighborhood of 200, the standard error is thus approximately 14, and the differences in total count at each time do not greatly exceed what might be expected on the basis of measurement error alone.

The coefficient of variation of repeated differential counts is approximately 14 per cent. Since the total erythrocyte precursor count is the product of the total cell count and the differential count, its coefficient of variation is approximately $\sqrt{7^2 + 14^2}$ per cent, or about 16 per cent. For counts in the neighborhood of 80, the standard error is thus approximately 13, and the differences in erythrocyte precursor total counts are appreciably larger than the estimated magnitude of the measurement error.

SUMMARY

The effects of 5 different types of serum upon erythropoiesis in bone marrow tissue culture have been tested, using a modification of the Mackaness and Smith

chamber. Eagle's medium and test serum in a 2:1 proportion were used as nutrient medium. Total and differential cell counts were done after 0, 12, 24, and, in one case, 36 hours of incubation at 37° C. Cultures sustained with normal rat serum showed a decrease in the number of erythrocyte precursors which is linear and predictable. Cultures grown in serum from rats rendered anemic by bleeding showed a marked increase in erythropoiesis after 12 hours of incubation. The same effect was seen in cultures grown with serum from rats that had been given cobaltous chloride (25 μm per 100 Gm. body wt.) 10 hours before the removal of the serum. When cobalt ion was added to normal serum in vitro to the same final concentration as found in the serum from injected rats, the resultant combination had no effect on erythropoiesis. When serum from rats rendered polycythemic was used, a marked decrease in erythropoiesis was noted after 12 hours of incubation.

These experiments give further evidence of the existence of a humoral factor (s) (erythropoietin) that stimulates erythropoiesis, and, in addition, establish that the substance(s) circulates in a form that is readily available to the bone marrow. The stimulatory effect of cobalt on erythropoiesis is through this hormone. In addition, there is also evidence that erythropoietin is present in serum from normal animals and also that the amount is decreased in serum from animals rendered polycythemic by transfusion.

We gratefully acknowledge the efforts of Dr. Paul Meier, Department of Statistics of The University of Chicago, in preparing the statistical analyses of these experiments and Dr. William Barclay of The University of Chicago School of Medicine for his instruction in the construction and use of the culture chambers.

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DETERMINATION OF REDOX ACTIVITY OF LEUKOCYTES AS A DIAGNOSTIC AID IN SYSTEMIC LUPUS ERYTHEMATOSUS

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IN 1953, Chaudhuri and Martin reported a metabolic alteration in leukocytes from patients with acute disseminated lupus erythematosus, as measured by the redox test.[1] The test is so named because it measures both reduction and oxidation. The leukocytes of patients with systemic lupus erythematosus (S.L.E.) exhibited a depression of activity as compared with the cells obtained from normal subjects. The leukocytes of normal individuals exhibited depression of redox activity following exposure to the plasma of patients with S.L.E.

The present study was undertaken to investigate the specificity of the redox reaction prior to attempts at identification of the plasma factor responsible for the altered metabolism.

MATERIALS AND METHODS

Studies were carried out on healthy medical students, patients with a variety of acute and chronic diseases, patients with systemic lupus erythematosus (untreated and treated with corticosteroids), and patients receiving hydralazine, as shown in Table I. Care was taken to include those entities which fall within the field of connective tissue disorders, those in which an allergic or hypersensitivity mechanism is postulated in regard to etiology, and those in which abnormal serum proteins are commonly found.

Redox activity was measured by the method of Chaudhuri and Martin.[1] Minutiae are added here, as minor technical variations influence the accuracy of the test.

1. Heparin: Liquaemin* sodium (heparin solution), 50 mg. per milliliter.

2. Saline: Sterile physiologic solution (0.9 per cent NaCl).

3. Dextran: A 5 per cent solution is prepared and autoclaved for 20 minutes at 20 pounds pressure (intrinsic viscosity 0.46). This solution will keep for 2 to 3 weeks, if refrigerated.†

4. Dye: 2,6-Dichlorophenol-indophenol. For stock solution, 20 mg. are dissolved in 200 ml. of saline. This solution may be kept for 2 months if refrigerated. For performance of the test, the dye-stock solution is diluted 1:6 in saline.‡

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*Organon, Inc., Orange, N. J.

†Commercial Solvents Corporation, Terre Haute, Ind.

‡Eastman Organic Chemicals, Rochester 3, N. Y.

TABLE I. SUMMARY OF CASES STUDIED

Systemic lupus erythematosus (prior to treatment)	22
Systemic lupus erythematosus (receiving steroids)	14
Discoid lupus erythematosus	9
Hypertension (6 receiving hydralazine)	11
Hypertension with hydralazine syndrome	5
"Febrile" rheumatoid arthritis	13
Rheumatoid arthritis	20
Marie-Strümpell spondylitis	5
Palindromic rheumatism	2
Acute rheumatic fever	2
Polyarteritis	2
Scleroderma	4
Arthralgia, bursitis, etc.	5
Ulcerative colitis	3
Psoriasis (1 with arthritis)	2
Pemphigus	1
Iritis	1
Reiter's syndrome	1
Osteoarthritis	20
Gout	2
Osteoporosis (etiology undetermined)	1
Allergic reactions (serum sickness)	3
Angioneurotic edema	1
Sarcoidosis	1
Multiple myeloma	2
Cirrhosis of the liver	2
Other diseases (pyelonephritis, sickle-cell anemia, pulmonary tuberculosis,, pulmonary interstitial fibrosis, duodenal ulcer, carcinoma of the colon, hypothyroidism, pregnancy, psychoneurosis)	18
Normal controls	84
Total	256

5. Dextran, dye, and saline *must* warm to room temperature *before* use.

6. All syringes, needles, and glassware must be siliconized.*

Ten milliliters of blood is placed in a rubber-stoppered test tube containing 0.2 ml. of heparin solution. The blood and heparin are mixed by whirling or inverting once. The "stopper area" of the tube is cleaned by wiping, and 2.5 ml. of dextran solution is added. (Dextran induces rapid sedimentation of the red cells and allows the separation of the white cells.) The tube is stoppered with a fresh stopper and mixed by whirling or inverting once. The stopper area of the tube is again wiped clean. The blood-dextran mixture is poured into a fresh tube, in order to remove any bubbles. The tube is then stoppered and allowed to stand upright for 30 minutes at room temperature. With a Pasteur pipette, the supernatant is transferred to a fresh tube, being careful to leave the sedimented red blood cells undisturbed. The supernatant is then mixed thoroughly without frothing, with a 5 ml. pipette. Five milliliters of this supernatant is placed into a fresh tube, then stoppered and incubated for 30 minutes in a 37° C. water bath. During this incubation period, a routine leukocyte count is done on the remainder of the leukocyte-platelet-plasma mixture. In the incubated mixture, the leukocytes are separated from the platelets and plasma by centrifugation at 50 g for 7½ minutes. The supernatant is drawn off with a Pasteur pipette attached to suction, taking care to leave the leukocytes undisturbed. The leukocytes are then resuspended in saline, adjusting the count to 7,500 per cubic millimeter. (A routine leukocyte count is performed on the white blood cell-saline suspension to assure a count of 7,500 per cubic millimeter. If the leukocytes are packed too tightly and do not resuspend easily, or if the count is too low, the results of the test are not accurate.)

*Siliclad: Clay-Adams, Inc., 141 East 25th Street, New York 10, N. Y.

One milliliter of resuspended cells is placed into each of 3 small tubes. One and five-tenths milliliters of diluted dye solution is placed into each of 2 small colorimeter tubes (12 by 75 mm.) and 1.5 ml. of saline into a third colorimeter tube.

First the saline and cells are mixed for the blank by pouring the saline into one of the tubes containing the cells and then back into the colorimeter tube. The instrument (Coleman Junior spectrophotometer, Bausch & Lomb spectrophotometer, etc.) is standardized with the blank. Reading is done at 600 mμ. The per cent transmission scale is used for the reading to facilitate rapidity. Standardization is repeated before each reading.

Once the instrument is standardized, the first dye-cell mixture is prepared by pouring the dye into the tube containing the cells and then back into the colorimeter tube. The timing is started immediately after mixing; the reading is done, and the dye-cell tube is placed in a 37° C. water bath. The spectrophotometer is restandardized and the duplicate set of cells and dye is mixed, timed, read, and incubated. The blank is then placed into the water bath. A few drops of mineral oil are poured into each tube to cover the top of the mixture and ensure anaerobiosis. The oil should run down the side of the tube and mixing with the contents of the tube must be avoided.

All tubes are read at 0, 5, 10, 20, and 30 minutes. The per cent of dye reduced is then determined. Calculations are made after conversion of per cent transmission to optical density.

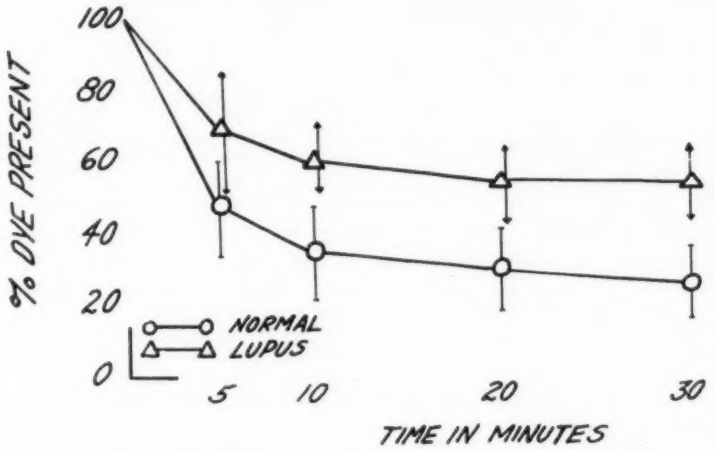

Fig. 1.—"Typical" normal curve (110 determinations) and "lupus" curve (72 determinations) with standard deviations. (Curves show the per cent of 2,6-dichlorophenol-indophenol, which has not been reduced by leukocytes.)

In this study all tests were done without knowledge of diagnosis beforehand. They were routinely run in duplicate with a normal control done in conjunction with each group of unknowns. Fig. 1 shows the typical normal curve as well as the typical "lupus" curve. Fig. 2 shows a comparison of typical curves obtained in this laboratory with those of Chaudhuri and Martin, and gives a clear indication of the reproducibility of their technique in our hands. Equally good correlation was obtained in all tests performed in our laboratory by different technicians.

L.E. cell preparations were performed on all individuals included in this study, using two different techniques.[2, 3]

RESULTS

The redox activity of leukocytes from 256 individuals was determined. Normal values (negative test) were obtained in 222. This group included acute and chronic diseases as well as 84 normal controls. Depressed redox activity (positive test) was found in 34 (Table II).

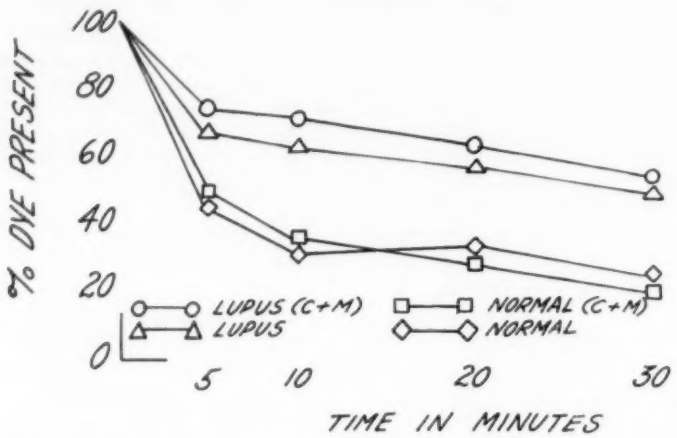

Fig. 2.—Comparisons of "typical" curves from this study (110 normal; 72 "lupus") with those of Chaudhuri and Martin (20 normal; 16 "lupus").

The conditions in which a positive redox test was obtained were: (1) systemic lupus erythematosus; (2) discoid lupus erythematosus; (3) connective tissue disorder, clinically compatible with either systemic lupus erythematosus or severe febrile rheumatoid arthritis, with consistently negative L.E. preparations; and (4) hydralazine syndrome. A total of 57 patients with these disorders were studied and 34 had a positive test.

TABLE II. SUMMARY OF CONDITIONS IN WHICH POSITIVE REDOX TEST WAS FOUND

CLINICAL DIAGNOSIS	NUMBER OF PATIENTS	REDOX TEST		L.E. CELLS POSITIVE
		POSITIVE	NEGATIVE	
Systemic lupus erythematosus (untreated)	14	14	0	14
Systemic lupus erythematosus (on steroid therapy)	8*	0	8	8
Discoid lupus erythematosus	9	3†	6	0
Differential diagnosis: S.L.E. or severe febrile rheumatoid arthritis	21	12‡	9	0
Hydralazine syndrome	5	5	0	3

*Six additional patients with positive tests prior to treatment had negative tests when receiving steroids.

†All had clinical evidence of dissemination.

‡Eight of these patients have subsequently had positive L.E. cell preparations or positive biopsy.

Fourteen patients of this group had systemic lupus erythematosus; L.E. cells were demonstrable at the time of initial study and all 14 had a positive redox test.

In a group of 9 patients with typical skin lesions of discoid lupus erythematosus, 3 positive redox tests were obtained. Clinically these 3 individuals had signs of systemic involvement such as leukopenia, albuminuria, fever, and elevated sedimentation rates, but negative L.E. cell preparations. In the remaining 6, there was nothing in the clinical course or laboratory tests to indicate dissemination.

Twenty-one individuals presented clinically as diagnostic problems with a clinical course compatible with severe febrile rheumatoid arthritis or systemic lupus erythematosus. L.E. cells were not demonstrable at the time of or prior to the performance of the redox test. Twelve of these 21 patients had a positive redox test. In 8 of these 12, the diagnosis of S.L.E. was substantiated later, not only by the clinical course, but also by the demonstration of L.E. cells in 6 of the patients, and by biopsies compatible with the diagnosis of S.L.E. in 2 others. In one of these patients the positive redox test was obtained 18 months prior to positive pathologic diagnosis. At the time the redox test was done, it was difficult to distinguish between the two entities by the clinical pattern of these 21 patients. Whereas 8 patients now have definite S.L.E., it has not as yet been possible to arrive at a more definitive diagnosis in the 13 other "febrile rheumatoids," 4 of whom have a positive and 9 a negative redox test. A few case summaries are included to illustrate the difficulty in clinical differentiation.

CASE REPORTS

Case 1.—L. E. is a 57-year-old white saleslady with a 2-month history of fever, bursitis of both shoulders, stiffness and swelling of the proximal interphalangeal joints of both hands, and generalized arthralgias and myalgias, accompanied by a weight loss of 35 pounds. Physical examination also revealed pleural effusion in the right chest. Laboratory studies revealed the following: hematocrit 36; sedimentation rate* 41 mm. per hour; white blood count 6,000 with normal differential; STS† negative; total protein 7 Gm. per cent with serum albumin 3.8 and globulin 3.3 Gm. per cent; gamma globulin 13 units‡; PPD No. 1 skin test positive. Scalene node biopsy revealed old tuberculoid granulomas. L.E. preparations were negative. Redox test was positive.

Case 2.—G. P. is a 50-year-old Negro woman with a 4-month history of fever, swelling, and pain in her ankles, knees, wrists, fingers, and elbows, bilateral iritis, and keratitis, accompanied by a weight loss of 20 pounds. Laboratory studies were as follows: hematocrit 39; sedimentation rate 46 mm. per hour; white blood count 7,050 with 5 per cent lymphocytes; STS negative; total protein 7.5 Gm. per cent with serum albumin 2.5 and globulin 5.0 Gm. per cent; gamma globulin 20 units; sheep-cell agglutination positive. L.E. preparations were negative. Redox test was positive.

Cases 1 and 2 are typical of the "febrile rheumatoids" in whom a positive redox test was found.

Case 3.—G. H. is a 28-year-old Negro stenographer with a 1-year history of pain and swelling of the wrists, fingers, knees, and ankles, following an episode of sore throat,

*All sedimentation rates (Wintrobe) corrected.
†STS = serologic test for syphilis.
‡Turbidimetric technique.[4]

fever, and left pleural pain. Chest x-ray, taken at time of onset, revealed a left pleural effusion with no evidence of tuberculosis. She had two episodes of parotid gland swelling for which specific etiology could not be determined. Laboratory studies were as follows: hematocrit 34; sedimentation rate 36 mm. per hour; white blood count 3,600 with 65 segmented forms, 5 band forms, and 30 lymphocytes; STS negative; total protein 8.6 Gm. per cent with serum albumin 3.0 and globulin 5.6 Gm. per cent; gamma globulin 21.5 units. L.E. preparations were negative. Redox test was negative.

CASE 4.—C. L. is a 20-year-old white female student with a 3-year history of fever, multiple joint swelling, and pain, accompanied by severe fatigue and weight loss. At times, an erythematous skin rash has been noted on the trunk and extremities. Past history revealed that she had acute rheumatic fever at the age of 11. The results of laboratory studies were as follows: hematocrit 34; sedimentation rate 38 mm. per hour; white blood count 8,450 with normal differential; STS negative; total protein 7.3 Gm. per cent with serum albumin 3.4 and globulin 3.9 Gm. per cent; gamma globulin 7 units; antistreptolysin-O titer 166 units; sheep-cell agglutination negative. L.E. preparations were negative, although on one occasion 2 "tart" cells were found. Redox test was negative.

CASE 5.—W. F. is a 72-year-old white man with an 18-month history of fever, joint pain, and swelling involving the knees, ankles, and proximal interphalangeal joints of the fingers, elbows, and shoulders, accompanied by a 40-pound weight loss. Laboratory studies revealed: hematocrit 36; sedimentation rate 44 mm. per hour; white blood count 10,500 with a slight shift to the left on differential; total protein 6.5 Gm. per cent with serum albumin 3.0 and globulin 3.5 Gm. per cent; gamma globulin 21 units; STS negative; sheep-cell agglutination positive. Chest x-rays have revealed a fibrotic infiltration in both lung fields which has gradually increased over a period of 12 months. Repeated studies for tuberculosis have been negative. L.E. preparations were negative. Redox test was negative.

These 3 cases are typical of the "febrile rheumatoids" in whom a negative redox test was found. The similarity of the clinical pattern with Cases 1 and 2 can be seen, the only significant difference being the results of the redox test. To date, L.E. cell preparations have remained negative in all 5 and there have been no differentiating features in the clinical course.

CASE 6.—L. F. is a 66-year-old white woman with a 1-year history of pain, stiffness, and swelling of the hands, shoulders, knees, and ankles, as well as stiffness of the neck, accompanied by fever, weight loss, and repeated episodes of nonspecific abdominal pain. Laboratory studies: hematocrit 28; sedimentation rate 36 mm. per hour; white blood count 5,200 with 76 segmented forms, 3 band forms and 21 lymphocytes; total protein 6.7 Gm. per cent with serum albumin 2.5; and globulin 4.2 Gm. per cent; gamma globulin 7.5 units; STS negative; sheep-cell agglutination negative. L.E. preparations were negative. Redox test was positive. Her course was progressively downhill. She developed marked anorexia and irritability. She was hospitalized 6 months later and restudied. X-rays showed bilateral lung involvement compatible with virus pneumonia or malignancy. Fluid was noted in both pleural cavities. All studies for tuberculosis were negative. L.E. preparations were now positive. Redox test remained positive. Therapy with corticosteroids was instituted. Redox test was repeated after steroids were administered and was negative. L.E. cell preparations were repeatedly positive, while on steroid therapy.

This case serves to illustrate the demonstration of a positive redox test in a "febrile rheumatoid" approximately 7 months prior to the demonstrations of positive L.E. cell preparations. In another instance, a positive redox test has been obtained 18 months prior to definitive diagnosis of S.L.E.

Six patients with S.L.E., postive L.E. preparations, and positive redox tests were examined again after they had been placed on corticosteroid

therapy. In each case the positive curve reverted to normal after steroid administration (Fig. 3).

In one patient the redox test was found to be negative 4 hours after a single intravenous dose of 50 mg. of hydrocortisone. As little as 5 mg. of prednisone per 24 hours was found to cause reversion of the abnormal redox activity to normal. Eight additional patients with S.L.E. and demonstrable L.E. cells were seen by us only after hormonal therapy had been started. All 8 patients had a negative redox test.

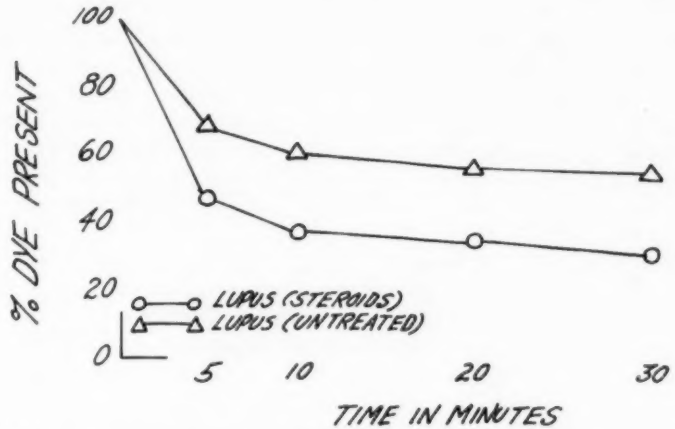

Fig. 3.—Redox curves of 6 patients with S.L.E. (18 determinations for each curve) before and after treatment with steroids. (See text.)

Five of 11 patients receiving hydralazine had a positive redox test. Four of the 5 had symptoms of the hydralazine syndrome at the time of study; L.E. cells were demonstrated in 3. The fifth individual was selected because he was receiving hydralazine in doses of 800 mg. daily. He was found to have positive L.E. preparations and a positive redox test, despite the absence of symptoms of the hydralazine syndrome. The 6 patients with a negative redox had negative L.E. cell preparations and no symptoms.

The dosage range of hydralazine was in excess of 300 mg. per day in 4 of the 5 patients. The other patient had had a severe hydralazine reaction 2 years prior to this study, following prolonged administration of the drug at levels of 600 mg. daily. No hydralazine was given in the 2-year interval. Following determination of redox activity, which was normal, a single 50 mg. dose of hydralazine was administered. The patient developed symptoms of the hydralazine syndrome and a positive redox test within 24 hours. L.E. cells were not demonstrable.

Depressed redox activity, symptoms of hydralazine syndrome and positive L.E. preparations, when present, reverted to normal in all instances when hydralazine was withdrawn (Fig. 4).

DISCUSSION

In recent years, a number of laboratory tests have been introduced in the field of connective tissue disease. The attempt is usually made to use these tests for a dual purpose, namely, as an aid in diagnosis and as a tool in the study of pathophysiologic processes. The data presented here indicate that the redox test may be a useful adjunct in the diagnosis and study of these diseases. Since the description of the L.E. cell by Hargraves and associates[5] many clinical syndromes have been identified as S.L.E., using the L.E. cell test as the principal criterion for diagnosis.

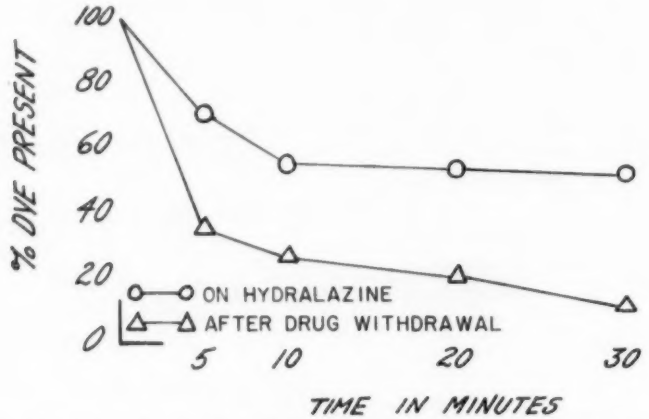

Fig. 4.—Redox curve of 3 patients with hydralazine syndrome (17 determinations for each curve) before and after (2 to 11 days) hydralazine withdrawal.

In comparison to the L.E. cell test, the redox test is technically more difficult to perform. Its use, therefore, solely as a diagnostic procedure in patients with an established clinical diagnosis of S.L.E. and positive L.E. cell preparation does not seem warranted. However, it may be of diagnostic value in those patients who have a clinical picture suggestive of S.L.E. but with negative L.E. preparations. Further, it may aid in the differentiation of those patients with diffuse systemic rheumatoid disease with positive L.E. cell preparations. The redox test has an advantage in accuracy, in that the test results are determined spectrophotometrically and are, therefore, not subject to the variations of technique and interpretations of suspicious or abnormal cell morphology. As stated by the editors of the Eleventh Rheumatism Review, "The status of the L.E. cell in rheumatoid arthritis is confusing because of the varied methods employed in performing the test, and because there is not complete agreement as to the characteristics which identify the L.E. cell."[6] Much the same comment might be made in regard to specificity. Positive L.E. cell preparations have been reported in penicillin sensitivity,[7] generalized moniliasis,[8] hydralazine syndrome,[9-11] diffuse systemic rheumatoid disease,[12] multiple myeloma,[13] pernicious anemia,[14] discoid lupus erythematosus,[14]

fungus contamination,[15] Senear-Usher pemphigus,[16] miliary tuberculosis,[17, 18] dermatitis herpetiformis,[14] leukemia,[19] and reaction to hydantoin,[20] as well as S.L.E. To the present time, the redox test has been found positive only in systemic lupus erythematosus (nonsteroid treated), in discoid lupus when there was evidence of dissemination, in patients with unclassified "febrile rheumatoid arthritis" whose clinical course was compatible with S.L.E., and in patients receiving hydralazine when symptoms of hydralazine reaction have developed. It would appear that a positive redox test is highly suggestive of S.L.E. Thus far, we have not encountered a patient who had untreated S.L.E., positive L.E. cell preparations, and a negative redox test.

The group of patients with unclassified "febrile rheumatoid arthritis" has been of particular interest. Application of the redox test has allowed division into two subgroups, those with a positive and those with a negative test. With respect to the clinical picture there were no differentiating features. In 8 of 12 patients who had a positive test, the diagnosis of S.L.E. was established at a later date, whereas all patients with a negative test have remained unclassified, both with regard to laboratory studies and clinical course.

As noted above, the redox test has also been found positive in patients receiving hydralazine, when symptoms of hydralazine reaction have developed. The significance of hydralazine reaction is not clearly defined. It has generally been considered that the reaction appears only after prolonged drug administration and, therefore, differs in mechanism from the usual type of hypersensitivity reaction to drugs.[21-23] In our experience there was no consistent correlation of positive redox test to dosage or duration of therapy. Thus far, the positive redox test correlated best with the development of symptoms of hydralazine syndrome. The one patient with a positive test who had no symptoms had positive L.E. preparations.

In further studies we have confirmed the observation that plasma from patients with systemic lupus erythematosus induces altered redox activity of normal leukocytes.[1] Pilot studies indicate that this same alteration is induced in normal leukocytes by plasma from patients with the hydralazine syndrome. The altered redox activity of leukocytes, therefore, appears to be attributable to a plasma factor rather than to an intrinsic defect of the white cell. It is of interest that a similar plasma factor can be postulated in both a drug-induced state and a naturally occurring disease. Further study will be necessary to learn more about this factor in both conditions.

Redox activity of leukocytes returns to normal when corticosteroids (cortisone, ACTH, hydrocortisone, prednisone, and prednisolone) are given to patients with S.L.E. or when hydralazine is withdrawn in patients with the hydralazine syndrome. Reversal of drug-induced abnormal redox activity to normal by factors other than drug withdrawal is now under study.

The change in redox activity of leukocytes reflects a profound alteration in cell metabolic structure.[1] At the present time the site and the reason for this alteration are not certain. As it is possible to induce this alteration in

normal leukocytes with the plasma of patients with S.L.E. and hydralazine syndrome, it will be of interest to attempt to isolate, identify, and further study this plasma factor and its effect on cells.

SUMMARY

The redox activity of leukocytes from 256 individuals has been studied. Depressed redox activity was found in 34. Abnormal activity was found in all of 14 cases of nonsteroid-treated typical systemic lupus erythematosus studied and in 8 additional patients with S.L.E. prior to definite diagnosis by currently acceptable methods. When positive redox tests were obtained in discoid lupus erythematosus, there were other data suggesting systemic involvement. In 4 of 13 "febrile rheumatoids," a positive redox test was demonstrated. Thus far no other differentiating features in the clinical course or laboratory findings have developed in this group of patients. Five patients receiving hydralazine demonstrated altered redox activity as well as symptoms of hydralazine reaction and/or positive L.E. preparations.

It is suggested that depression of redox activity of leukocytes, a positive redox test, may be specific for systemic lupus erythematosus and hydralazine reaction, and could, therefore, serve as a useful laboratory adjunct in early diagnosis.

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A MICROMETHOD OF EUGLOBULIN FIBRINOLYSIS IN PLASMA OF HUMAN SUBJECTS AND SMALL LABORATORY ANIMALS

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THERE exists a need for a micromethod to determine fibrinolytic activity. In an attempt to develop such a method, the macromethod described by Kowarzyk and Buluk[1] and particularly used by one of us[2-4] appeared to be suitable for adaptation to microvolumes. In this test the euglobulin fraction is precipitated by acetic acid and coagulated by calcium chloride. Fibrinolysis times of these euglobulin coagulates are then determined. This communication presents comparisons of plasma euglobulin fibrinolysis in human subjects, rabbits, guinea pigs, rats, and hamsters, using macro- and microvolumes of plasma.

METHOD

Fresh oxalated plasma (1 volume part 0.1 M ammonium oxalate and 9 volume parts blood) is obtained by centrifugation for 10 minutes at 1,500 r.p.m. In human subjects blood was drawn from the antecubital vein or the end phalanx of the finger, in which a large enough wound was inflicted to secure a free flow of blood. The blood was drawn from the ear vein and the heart of rabbits and from the heart of guinea pigs, rats, and hamsters.

The two different procedures used are described in the following sections.

A. Euglobulin Fibrinolysis in Human, Rabbit, Rat, and Hamster Plasma.—Tests in duplicate or triplicate are carried out as follows. To 0.05 ml. plasma in Pyrex tubes measuring 8 by 100 mm., add 0.9 ml. distilled water, containing 0.015 ml. of 1 per cent acetic acid. (The acidified water is prepared beforehand by mixing 90 ml. distilled H_2O with 1 ml. 1 per cent CH_3COOH.) The pH, which should be determined, is approximately 5.2. After these tubes have been kept for 30 minutes in a refrigerator at +4° C., they are centrifuged for 5 minutes at 1,500 r.p.m. The tubes are emptied of the entire supernatant, then placed upside down on filter paper to drain for about one minute, during which time the sediment adheres strongly to the wall of the tube. With the aid of a fine glass rod, the sediment is dissolved in 0.05 ml. borate solution of pH 9.0, which is composed of 9 Gm. sodium chloride and 1 Gm. sodium borate in 1,000 ml. distilled water. The tubes are then placed in a water bath at 37° C. and, 3 to 5 minutes later, 0.05 ml. of 0.025 M calcium chloride is added to the tubes which are maintained at a constant temperature. A coagulate forms within a few minutes. With all other plasma volumes employed, the volumes of the different solutions have to be adjusted accordingly. The tubes are kept in the water bath and observed every 5 minutes in order to see when the coagulate dissolves.

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B. Euglobulin Fibrinolysis in Guinea Pig Plasma or Serum.—To 0.05 ml. of plasma or serum, 1.0 ml. of acidified water is added. The pH of the mixture is 5.4. After the tubes have been kept for 18 hours in a refrigerator at 4° C., they are centrifuged. The sediment is then dissolved in 0.2 ml. borate solution and fibrinolysis is examined as soon as possible in a system composed of 0.1 ml. euglobulin, 0.2 ml. borate solution, 0.1 ml. thrombin (10 units per milliliter), 0.4 ml. of 0.2 per cent fibrinogen. The bovine fibrinogen employed is prepared by the method of Kekwick and associates[5] and contains 96 per cent clottable nitrogen. With all other plasma volumes employed, the volumes of the different solutions have to be adjusted accordingly. The tubes are kept in a water bath at 37° C. and observed every 15 seconds, the lysis of the coagulate being noted.

RESULTS

Table I shows duplicate results of euglobulin fibrinolysis employing from 0.5 to 0.02 ml. of human plasma from 4 healthy subjects. As can be seen, duplicate tests usually give rather close values of fibrinolysis time. Volumes of 0.02 ml. cannot be recommended since, as is also shown in Table I, in some instances the euglobulin solution does not coagulate. In all our tests euglobulin fibrinolysis times were found from 130 to 240 minutes.

TABLE I. EUGLOBULIN FIBRINOLYSIS IN DUPLICATE TESTS EMPLOYING VARYING AMOUNTS OF HUMAN PLASMA

PLASMA (ML.)	TUBE	FIBRINOLYSIS TIME (MINUTES) IN HUMAN SUBJECTS A	B	C	D
0.5	1	175	185	185	185
	2	185	175	185	195
0.3	1	170	190	200	185
	2	180	195	200	190
0.2	1	165	190	200	170
	2	175	185	200	185
0.1	1	180	200	240	185
	2	170	190	225	170
0.05	1	170	190	235	150
	2	175	185	200	150
0.02	1	—*	135	130	—*
	2	—*	145	135	—*

*Euglobulin solution did not coagulate.

TABLE II. COMPARISON OF EUGLOBULIN FIBRINOLYSIS IN PLASMA SECURED FROM HUMAN VENOUS AND CAPILLARY BLOOD

BLOOD	PLASMA (ML.)	FIBRINOLYSIS TIME (MINUTES) IN HUMAN SUBJECTS I	II	III	IV	V	VI
Venous	0.5	220	245	225	205	230	160
	0.05	245	195	240	210	225	180
Capillary	0.05	220	190	245	205	230	150

In Table II a comparison is given of fibrinolysis values in the plasma secured from the venous and capillary blood of 6 normal human subjects. As can be seen, there is no significant difference in fibrinolysis times between so-called "capillary" blood secured from the finger tip and venous blood.

In the rabbit both macro- and microvolumes were found to be applicable only if the following procedure is carried out. The centrifuged sediment is incubated in borate solution for about 2 hours in the refrigerator; otherwise, it does not dissolve readily in the borate solution. Using this modification, the lysis time employing varying volumes of plasma (shown in Table III) was found to be not as constant as with human or hamster plasma.

TABLE III. EUGLOBULIN FIBRINOLYSIS TIME IN TESTS EMPLOYING VARYING VOLUMES OF RABBIT PLASMA

PLASMA (ML.)	FIBRINOLYSIS TIME (HOURS) IN RABBITS						
	I	II	III	IV	V	VI	VII
0.5	4.4	5.0	4.2	4.6	6.3 IC	3.8	6.3
0.3	5.9	6.2	6.5 IC	3.3	5.8	6.1	4.7
0.2	3.8	4.5	6.5 IC	6.5 IC	3.8	6.3 IC	3.2
0.1	5.9	3.9	4.0	3.3	3.1	3.8	4.8
0.05	4.3	4.5	3.2	4.5	3.1	5.1	5.1
0.02	3.3	3.2	3.2	4.2	1.1	3.1	4.0

IC = Incomplete lysis; in all these instances, complete lysis was found after 21 hours.

In Table IV plasma euglobulin fibrinolysis employing 0.05 ml. plasma secured from blood drawn on different days from the hamster's heart shows that there is no significant difference in values. Not shown in Table IV are tests using varying volumes of 0.3, 0.1, 0.05, 0.03, and 0.01 ml. With the exception of the latter volume, all others exhibited similar fibrinolysis times. In the tests employing 0.01 ml. plasma, the euglobulin solution did not coagulate. Euglobulin fibrinolysis times varied from 75 to 140 minutes.

TABLE IV. EUGLOBULIN FIBRINOLYSIS MICROTESTS EMPLOYING PLASMA SECURED FROM BLOOD DRAWN ON DIFFERENT DAYS FROM THE HAMSTER'S HEART

HAMSTER	FIBRINOLYSIS TIME IN MINUTES	
	AUGUST 24	AUGUST 26
I	115	105
II	115	120
III	125	115
IV	110	125

Our findings with guinea pig plasma showed that the euglobulin solution does not coagulate. Guinea pig euglobulins show potent fibrinolytic activity, which suggests that their own fibrinogen is readily digested. This activity may be determined after the addition of thrombin and fibrinogen preparations to the euglobulin solution.

Table V shows the results of serum euglobulin fibrinolysis of 4 healthy and fasting guinea pigs. Volumes from 0.5 to 0.05 ml. guinea pig serum were employed. When guinea pig plasma is used instead, no significant difference in values has been obtained. For this animal, therefore, serum, which is more convenient for practical reasons, can be used instead of plasma.

The euglobulin fibrinolysis method cannot be recommended for testing rat plasma. The coagulates formed from rat plasma euglobulins are very resistant to fibrinolysis, since lysis times varied from 12 to 48 hours and more.

Since we found the use of 0.05 ml. plasma adequate in order to obtain results similar to those acquired with 0.5 ml. we adopted the 0.05 ml. plasma sample for the micromethod. If volumes of 0.02 ml. or even 0.01 ml. were used, the lysis times, wherever the euglobulin solutions could be coagulated, differed often from those obtained with the 0.05 ml. volume.

TABLE V. EUGLOBULIN FIBRINOLYSIS TESTS EMPLOYING VARYING VOLUMES OF GUINEA PIG SERUM

SERUM (ML.)	FIBRINOLYSIS TIME (SECONDS) IN GUINEA PIGS			
	I	II	III	IV
0.5	390	450	615	510
0.2	390	—	690	510
0.1	420	480	750	—
0.05	480	570	615	570

DISCUSSION

Kopec and Niewiarowski,[4] using the macromethod, found the normal fibrinolysis time for human subjects to be 219 minutes ± 5 minutes with a standard deviation ± 61 minutes. According to these authors, the fibrinolysis time in the euglobulin plasma fraction is influenced by many factors, such as the concentrations of fibrinolytic enzymes and fibrinogen, the pH with an optimum of 7.6, temperature, and the time interval elapsing between the collection of blood and the preparation of euglobulins. In normal subjects, this time interval does not appear to be significant. However, if fibrinolysis is activated, the blood samples should be preserved in an ice-water bath or the determination should be made immediately. Therefore, all tests were run directly after the blood was secured.

From observations by Milstone[6] and by Macfarlane and Biggs[7] it is well known that plasminogen is a chief component of the euglobulin fraction. However, the euglobulins are not completely free from antiplasmin and M. Niewiarowska found that the antiplasmin level influences the fibrinolysis time.[3] Also the activation of fibrinolytic enzymes in euglobulins is influenced by coagulation.[1, 2]

Although fibrinolysis in euglobulin and whole plasma does not always run parallel, as shown for instance by Ratnoff,[7a, 7b] fibrinolysis of the euglobulin fraction appears to be affected by factors similar to those in fibrinolysis of whole plasma in accordance with findings by Kopec and Niewiarowski[4] and Niewiarowska.[8] However, as experienced by these authors, the euglobulin method appears to be more sensitive and to give more consistent results than methods employing whole plasma. The euglobulin method can also be extended to the study of fibrinolysis in other animals.

Subsequent to these observations the reason for the failure of formation of a coagulate of guinea pig euglobulin has been found in studies of the fibrinolytic system in the guinea pig by Latallo, Niewiarowski, and Copley.[9] It was found that guinea pig plasma contains a plasmin-antiplasmin complex, which dissociates readily after dilution at pH 5.3 and that the euglobulin fraction

contains plasmin of very high potency. Fibrinolytic activity could be determined if small amounts (0.05 ml.) of guinea pig serum or plasma were used to precipitate the euglobulin fraction to which standard fibrinogen and thrombin solutions were added.

The euglobulin macromethod was widely used to examine fibrinolysis in human plasma in physiologic, as well as in various pathologic conditions in patients and, experimentally, in animals.[3, 9-12]

The application of the euglobulin fibrinolysis micromethod may prove to be useful in experimental studies with small laboratory animals[13] and by the use of subcutaneous capillary blood in clinical investigations of various pathologic conditions in children and in adults where venous blood withdrawal may be difficult.

SUMMARY

1. A micromethod employing 0.05 ml. ammonium oxalate plasma for euglobulin fibrinolysis was found to give consistent results in venous and capillary blood of human subjects and of the hamster's heart blood. The method represents an adaptation of a macromethod for the euglobulin fibrinolysis of Kowarzyk and Buluk.

2. The plasma euglobulin fibrinolysis times in healthy human subjects, using the micromethod, ranged from 150 to 245 minutes and in normal hamsters from 75 to 140 minutes.

3. The standardized euglobulin fibrinolysis test is not applicable to guinea pig plasma, since it constitutes a highly fibrinolytic system and appears to be activated during precipitation of the euglobulin fraction. Findings with a modification of the standard procedure, employing fibrinogen and thrombin solutions, are presented.

4. In normal rabbits, the method does not appear to be entirely satisfactory with regard to consistency of results.

5. The plasma euglobulin method is not recommended for rat plasma, because of the very low fibrinolytic activity found in this animal.

6. Plasma euglobulin fibrinolysis is discussed in connection with findings of the Polish investigators who introduced the macromethod.

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THE PHOTOMETRIC DETERMINATION OF THE SIALIC (*N*-ACETYLNEURAMINIC) ACID DISTRIBUTION IN CEREBROSPINAL FLUID

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NEURAMINIC acid, a complex amino acid sugar (its acetylated derivatives designated as sialic acid), is a functional group of many glycoproteins and glycolipids found in a variety of biologic fluids and tissues.[1-3] Its importance in neurologic problems is shown by the fact that it constitutes an integral part of the "gangliosides" which are complex lipids found in brain tissue.[4, 5] These gangliosides have been found to be increased in certain neurologic conditions, including Niemann-Pick disease and amaurotic familial idiocy (A.F.I.). In some instances they completely displace the normal cerebrosides and phospholipids of brain gray matter.[6, 7]

A number of investigators[8-11] have shown that *N*-acetylneuraminic acid is present in blood serum in such tightly bound combination with proteins that it is both nondialyzable and completely precipitable with trichloroacetic acid. The presence of sialic acid in cerebrospinal fluid (C.S.F.) has been demonstrated by Roboz and associates[12, 13] and by Uzman and Rumley.[14] However, the latter investigators showed that in the C.S.F. 60 to 80 per cent was present in a form which was dialyzable and not precipitable with trichloroacetic acid. Both groups of investigators employed methods which required large amounts (2 to 6 ml.) of C.S.F. for each analysis and use the relatively insensitive and nonspecific orcinol reaction for the colorimetric determination of its sialic acid content. The results reported by these experimenters[12-14] for individual cases held forth the promise that the C.S.F. might be expected to reflect certain pathologic disorders of the central nervous system to a greater extent than would the serum.[15] It was in order to test this hypothesis that the sensitive and specific diphenylamine reaction,[16] previously employed for serum analysis in our laboratory,[17, 18] was adapted to the quantitative determination of total sialic acid (TSA), protein-bound sialic acid, and the nonprotein-bound sialic acid, by difference, in cerebrospinal fluid. The method, as finally evolved, requires only 1.5 ml. of C.S.F. for the sialic acid determinations as well as for the total protein (TP) content.

The present paper deals with the results obtained with these procedures for the C.S.F. from a large number of patients with various neurologic diseases as well as for a control group of nonneurologic cases. In a number of

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instances, sialic acid values were determined for both serum and C.S.F. drawn simultaneously from the same individual. Evidence is presented for the possible diagnostic usefulness of such determinations in various neurologic diseases with special reference to amaurotic familial idiocy and related disorders.

METHODS

A. *Reagents and Apparatus.*—

1. Sodium chloride: 0.85 per cent solution.

2. Sodium hydroxide: 10 N solution.

3. Biuret reagent (modified after Gornall and associates[19]): In a liter volumetric flask containing about 500 ml. of distilled water, place 3.0 Gm. of $CuSO_4 \cdot 7\ H_2O$ and 12 Gm. of sodium potassium tartrate (Rochelle salt); shake until dissolved. Then, with shaking, add 300 ml. of 2.5 N NaOH. Add 2 Gm. KI, shake until dissolved, and dilute to volume with distilled water.

4. Total protein standard: Reconstitute a prepared serum standard, e.g., Versatol,* with 5.0 ml. of a solution composed of 10 per cent NaCl plus 1.0 per cent of disodium ethylenediaminetetraacetic acid. Refer to manufacturer's chart for protein content. Dilute 1.0 ml. to 100 ml. with 0.85 per cent NaCl in a volumetric flask. Keep refrigerated when not in use and discard when cloudy.

5. Trichloroacetic acid (TCA): 5.0, 7.5, and 10 per cent solutions were prepared by dilution from a 90 per cent stock solution.

6. Diphenylamine (DPA) reagent (1.0 per cent): Recrystallize diphenylamine twice from hot ethanol to remove impurities. Dissolve 1.0 Gm. in a mixture of 90 ml. of glacial acetic acid plus 10 ml. of concentrated sulfuric acid.

7. Sialic (*N*-acetylneuraminic) acid standard. A purified orosomucoid protein sample was prepared from outdated blood bank plasma by the procedure by Weimer and associates.[20] This preparation has a reported sialic acid content of about 11.2 per cent and was checked against a reference sample supplied by Dr. R. J. Winzler. For a 10 mg. per cent standard, dissolve 8.9 mg. of the orosomucoid preparation in 10 ml. of distilled water. Five and 2.5 mg. per cent standards were then prepared by dilution from this solution. The 10 mg. per cent standard solution will remain stable for about one week if kept frozen between runs.

8. Absolute ethyl alcohol.

9. Evaporation caps to fit 13 by 125 mm. Pyrex tubes.†

B. *Determination of Total Protein Content of Cerebrospinal Fluid.*—Pipette 1.0 ml. of C.S.F. samples into small test tubes calibrated at 2.0 ml. Add 1.0 ml. of 10 per cent TCA to each tube, and let stand at room temperature for 15 minutes. Centrifuge precipitates at 3,000 r.p.m. for 10 minutes. Decant off supernatant fluids and dissolve precipitates in one drop of 10 N NaOH by shaking tubes until precipitates are dissolved. Add 1.0 ml. of biuret reagent, and dilute to 2.0 ml. mark with 0.85 per cent NaCl solution. Mix well, let stand for 20 minutes, and read in a spectrophotometer at 530 mμ, or in a Klett-Summerson colorimeter, in a flat-bottom colorimeter tube, with a No. 54 filter against a saline blank (1.0 ml. 0.85 per cent NaCl plus 1.0 ml. biuret reagent) which has been set at zero. The TP standards are run simultaneously and in exactly the same manner as were the C.S.F. samples.

Calculations: Total protein (C.S.F.) mg. per cent $= \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times$ total protein standard (mg. per cent).

C. *Determination of Total Sialic Acid (TSA) Content of Cerebrospinal Fluid.*—Pipette 0.5 ml. of C.S.F. samples into 15 ml. centrifuge tubes. Add 1.0 ml. of 7.5 per cent TCA to each tube. Mix and cover tubes with glass evaporation caps. Heat tubes in a boiling

*Versatol is sold by Warner-Chilcott Laboratories, Morris Plains, N. J.
†Obtainable from Kopp Laboratory Supplies, 70-13 35th Rd., Queens 72, N. Y.

water bath for 15 minutes. Cool tubes in cold tap water and centrifuge precipitates* at 3,000 r.p.m. for 10 minutes. Pipette 1.0 ml. aliquots of the supernatant fluid into 13 by 125 mm. test tubes. Add 2.0 ml. of the DPA reagent and mix. Cover each tube with an evaporation cap and place in a boiling water bath for 30 minutes. Cool to room temperature in a cold water bath. A reagent blank (0.5 ml. water) and 0.5 ml. aliquots of 5 mg. per cent and 10 mg. per cent SA standards, in duplicate, are carried through exactly the same procedure as are the C.S.F. samples. The optical density values of the unknowns and standards are then read in a spectrophotometer at 530 mμ (or in a Klett photoelectric colorimeter with a No. 54 filter) against a reagent blank set at zero.

Calculations: Total sialic acid (C.S.F.) mg. per cent $= \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 10.0$ (or 5.0). Since the color reaction deviates from Beer's law, the standard used in making the calculations should be that which is closest to the unknown.

D. *Determination of Protein-Bound Sialic (N-Acetylneuraminic) Acid Content of Cerebrospinal Fluid.*—Into 15 ml. centrifuge tubes pipette 1.0 ml. aliquots of the C.S.F. and add to each tube 4.0 ml. of absolute alcohol. Mix contents and place tubes in a 65° C. water bath for 20 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r.p.m. for 10 minutes. Decant off supernatant fluid as completely as possible and break up precipitates by tapping bottom of the tube. Dry the precipitates by placing tubes in a boiling water bath until no alcohol odor can be detected. Add 1.5 ml. of 5.0 per cent TCA to each tube containing the alcohol precipitates. Pipette, in duplicate, into 15 ml. centrifuge tubes 0.5 ml. of distilled water (for reagent blank) and 0.5 ml. of 2.5 and 5.0 mg. per cent SA standards. Add 1.0 ml. of 7.5 per cent TCA to blank and standard tubes *only*. Mix and cover mouths of all tubes with evaporation caps. Heat all tubes in a boiling water bath for exactly 15 minutes. Cool tubes to room temperature in cold tap water and centrifuge at 3,000 r.p.m. for 10 minutes. Pipette 1.0 ml. aliquots of the supernatant fluid into 13 by 125 mm. test tubes. Add 2.0 ml. of DPA reagent and mix. The remainder of the procedure is exactly as described for the TNA determination in the preceding paragraph (C).

Calculations: Protein-bound sialic acid (mg. per cent) $= \frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 2.5$ (or 1.25).

E. *Total Protein and Total Protein-Bound, and Nonprotein-Bound Sialic Acid Content of the Cerebrospinal Fluid of Patients With Various Neurologic Diseases.*—C.S.F. was obtained by lumbar puncture from patients with a wide variety of neurologic and nonneurologic diseases. Bloody fluids and those with high cell counts were eliminated from these studies and all fluids were centrifuged prior to analysis. The total protein and total, protein-bound, and nonprotein-bound sialic acid values were then determined for each individual sample, usually in duplicate, by means of the procedures described above. In a number of instances serum total sialic acid and globulin sialic acid values were obtained by previously published procedures[17, 18] on blood samples drawn simultaneously with the C.S.F.

F. *Determination of "Nondialyzable" Protein-Bound Sialic (N-Acetylneuraminic) Acid Content of Cerebrospinal Fluid.*—Six pooled samples of C.S.F. were analyzed, in duplicate, for their protein-bound sialic acid content with the procedure described before in Paragraph D. Five milliliter aliquots of each of the 6 pooled samples were placed in cellophane bags and dialyzed for 16 hours in cold, running tap water in order to remove any nonprotein-bound sialic acid from the fluids. The contents of the bags were transferred to 5.0 ml. calibrated tubes and, after the addition of concentrated salt solution so as to give a final concentration of 0.9 per cent NaCl, adjusted to their original volume with water. This "nondialyzable" protein-containing C.S.F. fraction was then analyzed, in

*To conserve C.S.F., the dried precipitates obtained in this step can be dissolved in one drop of 10 N NaOH with shaking; the remainder of the procedure is carried out as described above for the T.P. determination except that the results are multiplied by 2.

duplicate, for its sialic acid with the procedure described in Paragraph C, except that 1.0 ml. aliquots were used. The results obtained in these runs are given in Table I together with the original total sialic acid values of each pooled sample.

TABLE I. COMPARISON OF PROTEIN-BOUND SIALIC ACID VALUES OBTAINED WITH ALCOHOL PRECIPITABLE FRACTION (AT 65°) AND WITH THE "NONDIALYZABLE" FRACTIONS OF THE SAME CEREBROSPINAL FLUID SAMPLES

SAMPLE NO.	TOTAL SIALIC ACID (MG. %)	PROTEIN-BOUND SIALIC ACID (ALCOHOL PRECIPITABLE FRACTION) (MG. %)	PROTEIN-BOUND SIALIC ACID ("NONDIAYZABLE" FRACTION) (MG. %)
1	6.9	1.2	1.4
2	8.9	1.8	1.8
3	6.8	0.9	1.1
4	5.7	0.9	1.0
5	10.5	1.6	1.6
6	6.2	1.1	1.2

RESULTS

For the patient control group, "presumed" normal cerebrospinal fluids were selected mainly from among nonneurologic cases having TP values between 20 to 45 mg. per cent. The mean value of 29.0 ± 1.6 obtained for this control group (Table II) is considerably lower than the value of 37.5 ± 1.2 previously reported from this laboratory.[15] However, the TP values were obtained by application of the biuret color reaction to the proteins *precipitated* by 5 per cent trichloroacetic acid. Some biuret-reacting glycoproteins and polypeptides are not precipitated in strongly acidic solutions[1, 14] and, in part, the decreased values may be due to the elimination of the faint turbidity which occurs upon the addition of many biuret reagents directly to the C.S.F.[21] Consequently the mean C.S.F. total protein value for the amaurotic familial idiocy group, i.e., 37.3 ± 3.2 mg. per cent with this modified procedure, is also lower than that reported in our previous paper,[15] i.e., 41.7 ± 2.5 mg. per cent.

As can be seen from the statistical data in Table II, both Group A (vascular diseases of the central nervous system) and Group B (demyelinating diseases) have somewhat elevated TP values, whereas Group D (cases with extensive brain damage) exhibits markedly elevated C.S.F. total protein values. These groups were included in this study in order to permit an investigation of the C.S.F. sialic acid distribution over a wide range of TP values and the inclusion of many different types of neurologic disorders. In agreement with the reported results of Uzman and Rumley[14] in 49 samples of human C.S.F., those obtained in this study for the 46 cases in Groups A, B, C, and E show that 60 to 80 per cent is present in a form which is not bound by the C.S.F. proteins. Group D, the high TP group, shows only 40 to 70 per cent of the total sialic acid present to be in an unbound state.

The mean C.S.F. total sialic acid for the control group, i.e., 5.1 ± 0.3 mg. per cent obtained with the diphenylamine method, is markedly increased over the range of 1.1 to 3.4 mg. per cent reported by Uzman and Rumley[14] with the orcinol procedure of Böhm and associates.[22] A similar difference of values for serum total sialic acid has also been found between the two procedures,

TABLE II. GROUP STATISTICAL DATA OF CEREBROSPINAL FLUID PROTEINS AND SIALIC ACID VALUES IN 42 PATIENTS WITH VARIOUS NEUROLOGIC DISEASES AS COMPARED WITH A CONTROL GROUP OF 15 "PRESUMED" NORMAL SUBJECTS

DISEASE GROUPS	TOTAL PROTEIN (MG. PER CENT)	TOTAL SIALIC ACID (MG. PER CENT)	PROTEIN-BOUND SIALIC ACID (MG. PER CENT)	NONPROTEIN-BOUND SIALIC ACID (MG. PER CENT)	TOTAL SIALIC ACID / TOTAL PROTEIN (PER CENT)	PROTEIN-BOUND SIALIC ACID / TOTAL SIALIC ACID (PER CENT)
A. Vascular diseases of central nervous system (parkinsonism, cerebrovascular disorders, etc.) 11 cases	34.7 ± 3.8* 24.1 – 45.3† N.S.‡	6.7 ± 0.4 5.6 – 7.8 <0.01	1.4 ± 0.08 1.2 – 1.6 N.S.	5.3 ± 0.4 4.2 – 6.4 N.S.	20.9 ± 1.8 15.9 – 25.9 N.S.	20.9 ± 1.2 17.5 – 24.3 N.S.
B. Demyelinating diseases (multiple sclerosis, Schilder's disease, etc.) 9 cases	34.8 ± 3.9* 33.7 – 35.9† N.S.‡	6.4 ± 0.8 4.1 – 8.7 N.S.	1.1 ± 0.1 0.8 – 1.4 N.S.	5.6 ± 0.7 3.6 – 7.6 N.S.	19.6 ± 2.0 13.9 – 25.3 N.S.	16.7 ± 1.3 13.0 – 20.4 N.S.
C. Amaurotic familial idiocy (Tay-Sachs disease) 12 cases	37.3 ± 3.2* 28.3 – 46.3† N.S.‡	3.7 ± 0.4 2.6 – 4.8 <0.01	0.8 ± 0.06 0.6 – 1.0 <0.01	3.0 ± 0.3 2.2 – 3.8 <0.01	10.2 ± 0.7 8.2 – 12.2 <0.01	21.4 ± 1.7 16.6 – 26.2 N.S.
D. Cerebral degeneration—extensive (hydrocephalus, Niemann-Pick, craniopharyngioma, etc.) 10 cases	151 ± 22.7* 87.2 – 214.8† <0.01‡	8.5 ± 1.2 5.1 – 11.9 <0.01	2.9 ± 0.4 1.8 – 4.0 <0.01	5.6 ± 1.1 2.5 – 8.7 N.S.	6.4 ± 1.3 2.8 – 10.0 <0.01	36.9 ± 4.3 24.7 – 49.1 <0.01
E. Control group (nonneurologic cases) 15 cases	29.0 ± 1.6* 24.6 – 33.4†	5.1 ± 0.3 4.3 – 5.9	1.2 ± 0.07 1.0 – 1.4	4.0 ± 0.3 3.2 – 4.8	17.9 ± 0.7 16.0 – 19.8	24.0 ± 2.2 17.9 – 30.1

*Mean ± standard error (S.E.).
†Estimated range of population = mean ± tx (S.E.).
‡Probability at 0.01 level. N.S. = not significant.

i.e., 44 to 50 mg. per cent for the orcinol method[22] and 74 to 81 mg. per cent for the diphenylamine.[17, 18] Since the high order of specificity of the diphenylamine reaction for 2-desoxyhexoses and hexals has been stressed by several investigators,[16, 23] the difference in the results reported between the two procedures might either be due to the lesser specificity, or sensitivity, of the orcinol reagent or to the presence of unknown diphenylamine-reacting substances in both serum and C.S.F. In any event, significantly lower values for the C.S.F. total sialic acid are obtained for Group C, the amaurotic familial idiocy cases, whereas values almost twice the normal mean have been obtained for such disorders as amyotrophic lateral sclerosis, Parkinson's disease, and cerebral thrombosis, although the TP results are still in the normal range.

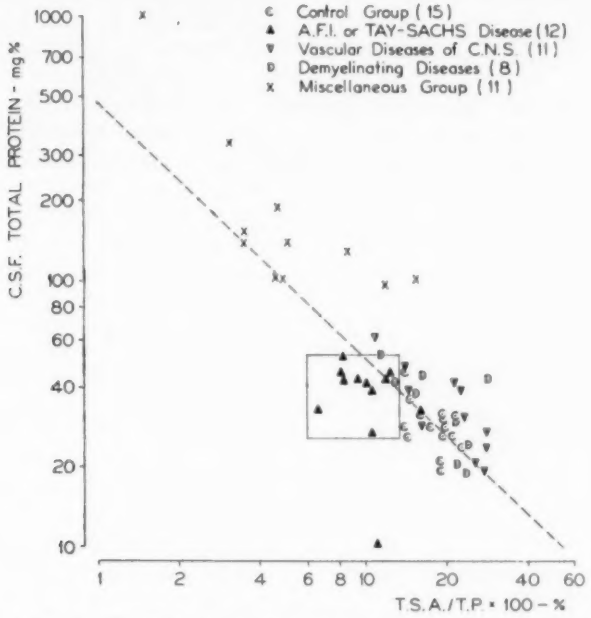

Fig. 1.—Variation of TSA/TP percentage ratio with increasing TP concentration of the C.S.F. of 44 patients with various neurologic diseases and 15 control cases with nonneurologic disorders. The dotted line represents the median values of the control group.

The relationship between the total sialic acid (TSA) and total protein (TP) of the C.S.F. for the 57 individual cases studied is illustrated graphically in Fig. 1 where the data are presented as a log-log plot of TP against TSA/TP ratio values. If the TSA/TP results varied in a manner exponential to the log of the TP values, then most of the points should fall along, or be adjacent to, the dotted line. While there is a general trend in the direction of lower TSA/TP ratios with higher TP values for most of the values, there are two notable exceptions among the groups of neurologic cases investigated. For

Group D, with extensive brain damage and high protein values, there is an increase in the total sialic acid that is disproportionate to that of the TP so that most points in this group lie above the line. In at least one instance the TSA/TP value approaches 1.0 per cent, the approximate relationship of the TSA/TP ratio in normal serum. Conversely, there appears to be a decrease in the total sialic acid in relation to the TP for the amaurotic familial idiocy group (Group C), many of the points falling into a definite region below the line (cf. Fig. 1, boxed area). The decrease in total sialic acid found in this disease is primarily due to the lower nonprotein-bound sialic acid values (see Table II).

The mean value of 1.2 ± 0.07 mg. per cent obtained for the protein-bound sialic acid fraction of the C.S.F. with the diphenylamine procedure is again considerably elevated above that found for the trichloroacetic acid precipitable fraction by Roboz and associates,[12] of 0.24 ± 0.3 mg. per cent. In a later publication Roboz and co-workers[13] employed alcohol precipitation at 4° C. for 24 hours as an improved procedure for protein precipitation from the C.S.F., requiring only 2.0 ml. per determination, prior to the application of the orcinol method for the determination of this fraction. They also reported increased protein-bound sialic acid levels in C.S.F. of patients with various neurologic disorders and the same normal value with alcohol precipitation as they had obtained with trichloroacetic acid precipitation. Uzman and Rumley[14] give a few individual values from which the protein-bound sialic acid obtained with trichloroacetic acid precipitation can be compared with the values obtained after dialysis for the same spinal fluids. The acid-precipitated protein-bound sialic acid varies from 0.22 to 0.47 mg. per cent whereas that found for the nondialyzable fraction varied from 0.45 to 0.81 mg. per cent for the same fluids. These findings have been confirmed in our laboratory and led us to question the completeness of protein precipitation by cold alcohol (4° C.) as a necessary step for the quantitative determination of the protein-bound sialic acid. It was found experimentally that only alcohol precipitation with heat at 65° C. for 30 minutes, as suggested by Südhof and associates,[24] gave results for C.S.F. protein-bound sialic acid that were comparable to those obtained with the nondialyzable protein fraction (see Table I). The procedure for the determination of this fraction, as described here, is less time consuming than those previously proposed and requires only 1.0 ml. of C.S.F. per determination.

Examination of the statistical data in Table II shows that there is a significant decrease in the protein-bound sialic acid values for Group C (amaurotic familial idiocy) as compared to controls. Only one case out of 12 in this group gave a value in excess of 1.0 mg. per cent. Markedly increased C.S.F. protein-bound sialic acid results are seen principally in patients of Group A, e.g., parkinsonism and cerebrovascular disorders.

The results given in Table III show some of the individual data obtained in various neurologic conditions in which the distribution of the sialic acid was studied simultaneously in C.S.F. and serum. The serum results again illustrate the unusually high values of the globulin sialic acid/total sialic acid

percentage ratio found in amaurotic familial idiocy as compared to most other neurologic diseases.[18] These results also illustrate the wide range of values for total sialic acid, TSA/TP (per cent), and protein-bound and nonprotein-bound sialic acid which are found in pathologic spinal fluids. Again the cases of amaurotic familial idiocy, as a group (Table II), show significant decreases in the TSA/TP ratio and in the protein-bound sialic acid values of the C.S.F. as compared to other neurologic disorders which may aid in the diagnosis and in the elucidation of the nature of this invariably fatal childhood disease.

TABLE III. INDIVIDUAL DATA OF BOTH CEREBROSPINAL FLUID AND SERUM SIALIC ACID DISTRIBUTION LEVELS IN PATIENTS WITH VARIOUS NEUROLOGIC DISEASES

		CEREBROSPINAL FLUID					SERUM		
PATIENT	DIAGNOSIS	TP (MG. %)	TSA (MG. %)	PBSA (MG. %)	NPBSA (MG. %)	TSA / TP %	TSA (MG. %)	GSA (MG. %)	GSA / TSA %
1. S. B.	Cerebral palsy	34.5	4.8	0.8	4.0	13.9	81.7	56.8	69.5
2. S. L.	Cerebral vascular disease with hemiplegia	47.7	6.7	1.5	5.2	14.0	76.8	43.6	56.7
3. I. S.	Postpolio	23.9	5.4	1.8	3.6	22.6	87.3	54.5	62.4
4. B. E.	Amyotrophic lateral sclerosis	43.0	12.4	1.7	10.7	28.8	—	—	—
5. J. B.	Multiple sclerosis	44.2	7.2	1.0	6.2	16.4	82.4	46.7	56.7
6. L. W.	Spina bifida	101.0	4.9	2.8	2.1	4.9	—	—	—
7. N. E.	Hydrocephalus, congenital	99.0	11.6	4.8	6.8	11.7	—	—	—
8. S. R.	Craniopharyngioma	138.0	4.8	2.6	2.2	3.5	—	—	—
9. J. M.	Schilder's disease	55.5	4.5	0.9	3.6	8.1	97.6	67.4	69.0
10. B. S.	Niemann-Pick disease	213.0	5.9	2.4	3.5	3.0	61.4	36.5	59.0
11. S. S.	A.F.I., infantile	25.0	3.2	0.8	2.4	12.7	101.0	67.4	67.0
12. V. R.	A.F.I., infantile	42.8	5.2	0.8	4.4	12.1	101.0	67.8	67.1
13. H. D.	A.F.I., infantile	47.5	4.1	1.1	3.0	8.6	107.0	74.6	69.7
14. K. S.	A.F.I., infantile	45.2	3.4	0.9	2.5	7.6	108.2	68.0	63.0
15. L. D.	A.F.I., juvenile	30.9	5.4	0.7	4.7	17.3	68.8	43.1	62.7
16. B. S.	Normal	42.8	4.8	1.3	3.5	11.1	61.7	33.7	54.6

Key to abbreviations: TP = total protein; TSA = total sialic acid; PBSA = protein-bound sialic acid; NPBSA = nonprotein-bound sialic acid; GSA = globulin sialic acid.

DISCUSSION

Studies of spinal fluid sialic acid distribution as applied to specimens obtained from *individual* patients have been few in number[12-14] because of the lack of adequate quantitative methods requiring but small amounts of C.S.F. It was for this reason that the diphenylamine colorimetric reaction for serum sialic (or *N*-acetylneuraminic) acid previously published[17, 18] was employed in this investigation of the total, protein-bound, and nonprotein-bound sialic acid in C.S.F. Careful investigation of the characteristic purple diphenylamine color in serum by Hess and associates[16] has shown it to be characteristic of a 2-desoxyhexose, or hexal, structure and that this structure, or a common derivative of it, accounts for the adsorption maxima at 520 mμ. A desoxyketose structure in neuraminic acid (or its derivatives), as proposed by Gottschalk,[3, 25] would also account for the color reaction of these substances with the diphenylamine reagent. Optical density measurements at or near this wave length of the diphenylamine color of the C.S.F. were, therefore, assumed to be a quantitative measure of their sialic acid content. The kind

of sialic acid present in most biologic substances in which it is firmly bound to proteins is believed to be *N*-acetylneuraminic acid.[2, 3, 9] However, the nature of the neuraminic acid derivative present in C.S.F. which is dialyzable, and not bound to protein, has not as yet been established although it constitutes 60 to 80 per cent of the total sialic acid of this important biologic fluid.[14]

This investigation was undertaken in the belief that quantitative determinations of the sialic acid content of the C.S.F. would not only give diagnostic information but might also provide a clearer insight into the mechanism by which large amounts of sialic acid-containing gangliosides are deposited in brain gray matter in some lipoidoses.

SUMMARY

1. Methods are presented for the quantitative determination of the total sialic acid (TSA), protein-bound sialic acid, and nonprotein-bound sialic acid of the cerebrospinal fluid (C.S.F.) which require a total of 1.5 ml. of fluid, inclusive of the total protein (TP).

2. The colorimetric measurements are based on the relatively specific and sensitive diphenylamine (DPA) reaction which previously had been applied only to serum. Complete separation of the protein-bound from the nonprotein-bound sialic acid is achieved by use of alcohol precipitation with heat (30 min. at 65° C.).

3. Individual values were obtained for the C.S.F. of 42 patients with various neurologic diseases, as well as for a control group of 15 nonneurologic cases with normal TP values. Contrary to the findings in serum or tissues, 60 to 80 per cent of the TSA is present in a nonprotein-bound form.

4. Log-log plots of TSA/TP versus TP show a general trend in the direction of lower TSA/TP ratios with higher TP values for the control group (Group E) and for most neurologic conditions. Exceptions to this relationship were the cases with marked cerebral degeneration and high TP values, and the group of cases with amaurotic familial idiocy which showed highly significant differences by group statistics.

5. The general pathologic and diagnostic significance of sialic acid is discussed.

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THE EFFECT OF UREA ON CEREBROSPINAL FLUID PRESSURE IN MONKEYS BEFORE AND AFTER BILATERAL NEPHRECTOMY

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MANY hypertonic solutions have been used for reduction of cerebrospinal fluid pressure since the original work of Weed and McKibben.[1] Their undesirable features have markedly limited their use. The possibility of clinical application of urea was suggested by Fremont-Smith and Forbes in 1927[2]; however, their suggestion was not followed until 1954 when Javid and Settlage administered urea to human subjects.[3] More than 350 patients have received this agent for reduction of intracranial or intraocular pressure.[4, 5] About one hundred studies have been carried out in Rhesus monkeys.[6] The efficacy of urea has been compared with other hypertonic solutions as well as mercury and Diamox (acetazoleamide). Urea was found to be superior in effectiveness without producing adverse reactions. These findings added impetus in carrying out the following experiments.

The osmotic effect of hypertonic solutions and the inverse relationship between cerebrospinal fluid pressure and urinary output are well known.[1, 7] Experience with urea substantiates this point.[8-10] There is evidence that this reduction is presumably due to osmotic pressure change although it is possible that other factors are involved. The exact mechanism of action seems more complicated.[11] To determine the diuretic role of urea in lowering cerebrospinal fluid pressure, we investigated the effect of urea and other hypertonic agents in monkeys before and following nephrectomy.

MATERIALS AND METHODS

Five female Rhesus monkeys *(Macaca mulatta)* weighing 1.80 to 6.25 kilograms were the subjects. The animals were anesthetized with intramuscular pentobarbital, 42 mg. per kilogram body weight initial dose, and placed in a prone position. A cisternal needle was connected to a specially built open-end manometer consisting of polyethylene tubing (No. 100). Cerebrospinal fluid pressures were recorded every 5 minutes for 8 to 71 hours. Three monkeys received urea in 30 per cent concentration and 1 monkey sucrose in 50 per cent concentration; 1 received 50 per cent dextrose before and after nephrectomy followed by other hypertonic solutions. These solutions were given on an equimolar basis with one gram urea per kilogram body weight. A predetermined precise amount of urea, sucrose, and glucose was injected intravenously in divided doses every 30 seconds, in the course of 30 minutes to individual animals. In one study the duration of injection was 60 minutes. Rapid administration was also carried out as indicated in the figures.

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RESULTS

Fig. 1 demonstrates an inverse relationship between cerebrospinal fluid pressure and volume of urine excreted following the administration of urea before and after unilateral nephrectomy.

It will be seen that maximum reduction of cerebrospinal fluid pressure corresponds with a maximum diuresis. However, the pressure drop is more pronounced and better sustained in the unilaterally nephrectomized monkey where diuresis is decreased.

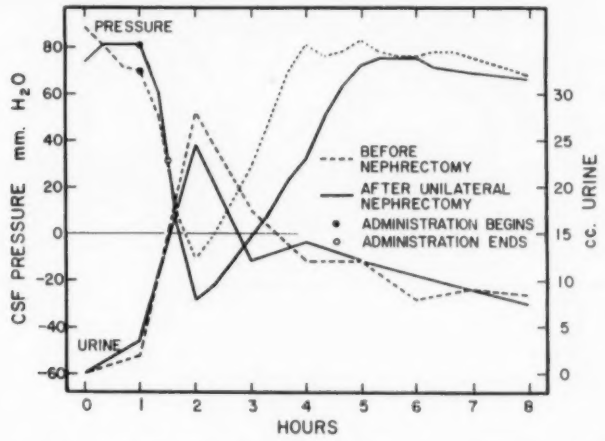

Fig. 1.—Illustrated is the inverse relationship between cerebrospinal fluid pressure and urinary excretion, before and after unilateral nephrectomy, following administration of identical amounts of urea (30 per cent) in Monkey 104. Lowest cerebrospinal fluid pressure corresponds with maximum diuresis, but pressure drop is greater and better sustained in unilaterally nephrectomized monkey.

Fig. 2 shows the effect of urea when bilateral nephrectomy was done simultaneously. It can be seen that fall in cerebrospinal fluid pressure following nephrectomy was greater and more sustained. This monkey tolerated bilateral nephrectomy and was alert for 3 days, then failed rapidly and died on the fourth postoperative day.

In Fig. 3 the cerebrospinal fluid pressure changes following unilateral and bilateral nephrectomy are compared with the pressure before nephrectomy. It will be noted that pressure drop following unilateral nephrectomy is greater than in the normal animal and the drop is still greater after bilateral nephrectomy. At the twenty-eighth hour after the beginning of the experiment, a similar amount of urea was administered again. The pressure dropped promptly and reached –80 mm. H_2O one hour after completion of urea administration. The animal was sacrificed 30 minutes later. Blood urea nitrogen was 182 mg. per 100 ml., cerebrospinal fluid urea nitrogen was 125 mg. per 100 ml. It is significant that, even in this bilaterally nephrectomized monkey, the

discrepancy between the urea nitrogen level of blood and cerebrospinal fluid corresponds to what is always seen in a nonnephrectomized subject, i.e., during the first few hours after administration of urea, cerebrospinal fluid urea nitrogen levels lag behind that of blood.[3, 4]

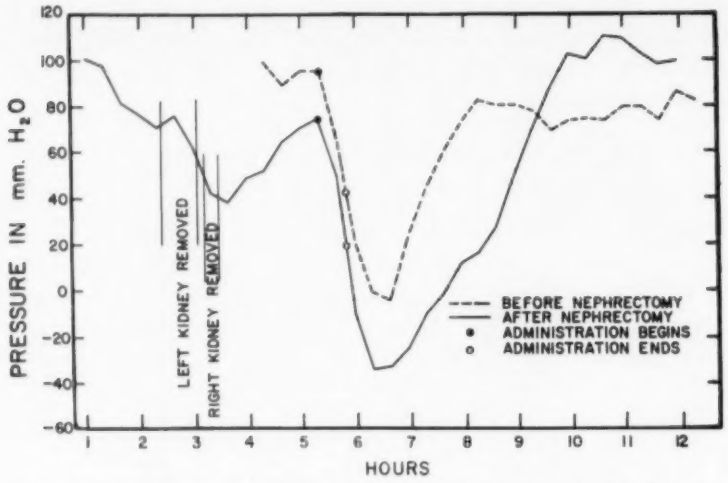

Fig. 2.—The cerebrospinal fluid pressure changes are demonstrated after urea administration in Monkey 88 before and following removal of both kidneys. Sufficient time was allowed following bilateral nephrectomy for cerebrospinal fluid pressure to return to prenephrectomy level before injection of urea.

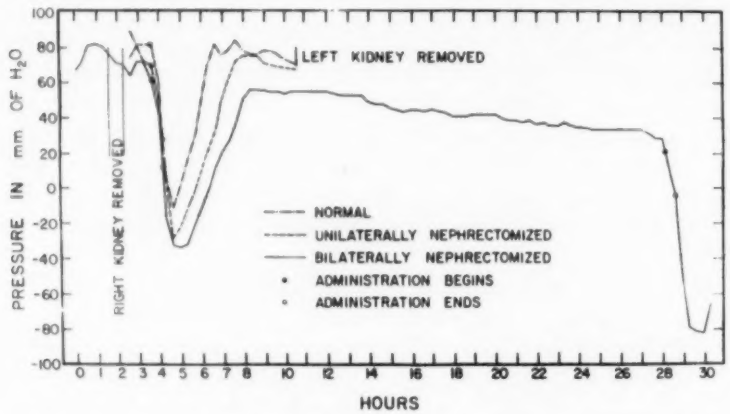

Fig. 3.—The cerebrospinal fluid pressure changes after urea administration in Monkey 104 before nephrectomy, following unilateral nephrectomy, and following bilateral nephrectomy are illustrated.

Fig. 4 illustrates pressure reduction following urea administration before and after nephrectomy. It can be seen that cerebrospinal fluid pressure reduction is more profound and better sustained after bilateral nephrectomy. At

23 hours postnephrectomy a similar dose of urea was injected rapidly, intraperitoneally, and produced a pressure drop to –89 mm. H_2O. At 29 hours postnephrectomy an identical amount was injected rapidly into the stomach and caused pressure to fall to 6 mm. below atmospheric level. The monkey died at 41 hours postnephrectomy.

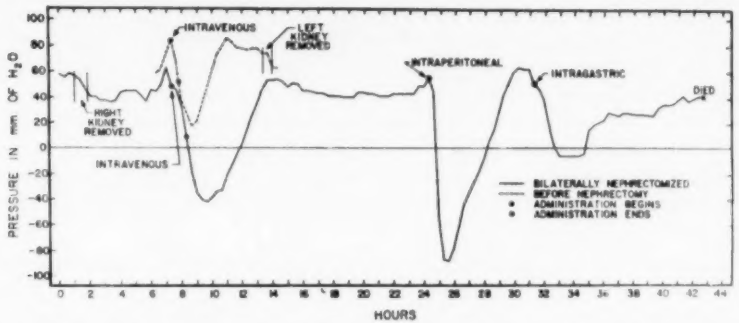

Fig. 4.—The cerebrospinal fluid pressure changes following urea administration, before and after bilateral nephrectomy, are shown for Monkey 92. The duration of injection after bilateral nephrectomy was 60 minutes.

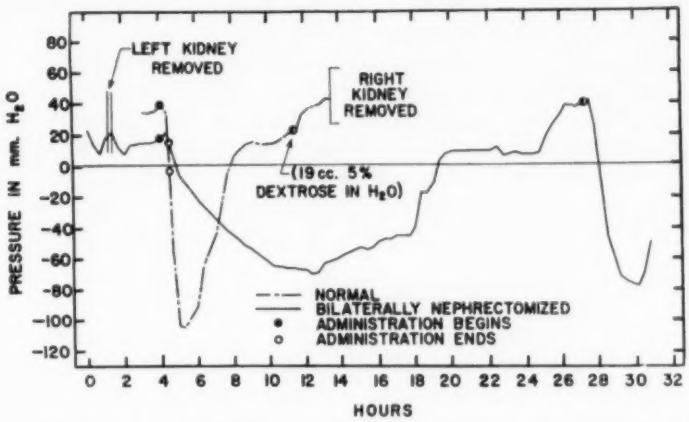

Fig. 5.—Monkey 113. Illustrated are cerebrospinal fluid pressure changes following 50 per cent sucrose administration, equimolar with one gram per kilogram urea.

Fig. 5 shows pressure changes following 50 per cent sucrose administration, equimolar with one gram per kilogram urea. The fall in cerebrospinal fluid pressure is slow after bilateral nephrectomy, but sustained for approximately 15 hours. At 27 hours an identical amount of sucrose was injected rapidly. There was a decrease of cerebrospinal fluid pressure to –78 mm. H_2O. The animal was sacrificed at 31 hours.

Some additional observations were made in one monkey (No. 109) following administration of 50 per cent dextrose equimolar with one gram per kilogram urea before and after nephrectomy. It was noted that when dextrose

was injected slowly during an interval of 30 minutes, reduction of cerebrospinal fluid pressure was minimal, but after bilateral nephrectomy the pressure drop was more marked. The lowering of cerebrospinal fluid pressure following rapid administration of dextrose (5 minutes) were identical before and after nephrectomy.

DISCUSSION

Findings in this report indicate that the effect of urea in reducing cerebrospinal fluid pressure is increased after nephrectomy. Thus, diuresis does not play an important role in lowering the intracranial pressure. Of interest are the following: the prompt and consistent fall in cerebrospinal fluid pressure in nephrectomized monkeys (Nos. 104 and 113) after similar amounts of urea and sucrose were repeatedly given, the effectiveness of urea in a nephrectomized monkey (No. 92)—whether urea was injected intravenously, intraperitoneally, or in the stomach.

The manner of fall in cerebrospinal fluid pressure in the nephrectomized monkey following sucrose, dextrose, and urea seems to be different. In the case of sucrose, there is the slow initial fall in cerebrospinal fluid pressure as compared to rapid fall with urea and dextrose. In the case of dextrose, the minimal discrepancy in cerebrospinal fluid reduction between pre- and post-nephrectomy studies is probably due to the fact that this substance is metabolized, whereas sucrose and urea are not.

It would appear that prolonged maintenance of cerebrospinal fluid pressure reduction following urea administration in nephrectomized monkeys is due to prolonged osmotic pressure differential between blood and cerebrospinal fluid. The latter is due to the fact that urea cannot be eliminated via kidneys; consequently, it remains in a high concentration in the blood.

Clinically, whether inhibiting urinary output by use of drugs would increase the effectiveness of urea in reducing intracranial pressure, raises a question worthy of investigation.

SUMMARY

In order to determine the role of diuresis in reducing cerebrospinal fluid pressure following the administration of urea, a new approach is described. Bilateral nephrectomy was carried out in 5 Rhesus monkeys. In each instance, following administration of urea and other hypertonic solutions, the fall in cerebrospinal fluid pressure occurs and is better maintained in the absence of urinary excretion.

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LABORATORY METHODS

SPECTROPHOTOMETRIC DETERMINATION OF TOLBUTAMIDE IN PLASMA

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KALAMAZOO, MICH.

THE hypoglycemic agent tolbutamide (1-butyl-3-*p*-tolylsulfonylurea, Orinase®) can be quantitatively determined in serum or plasma by ultraviolet spectroscopy utilizing the intense absorption band at 228 mμ. Spingler and Kaiser[1] lyophilized the serum and extracted the tolbutamide from the dry residue with acidified ethyl acetate. A correction for the blank value of serum (no tolbutamide present) was made by obtaining a serum sample prior to tolbutamide administration, i.e., a zero hour sample. Forist and associates[2] used chloroform to extract the tolbutamide from plasma acidified to pH 4.5 to 4.8. A small amount of activated charcoal was used to decrease the blank value but a zero hour sample was recommended. Bladh and Nordén[3] also used the absorption band at 228 mμ to determine tolbutamide in serum. The serum was acidified with hydrochloric acid and chloroform was used as the extracting solvent. A column of calcium carbonate or sodium bicarbonate was used to reduce the blank value. A zero hour sample was specified.

Spingler[4] described a method based upon visible spectroscopy in which tolbutamide was extracted from acidified serum with amyl acetate. The tolbutamide was hydrolyzed to butylamine by heating at 150° C. for 5 minutes. The butylamine was then determined by adding 2,4-dinitrofluorobenzene and determining the absorbance at 380 mμ. A zero hour sample was not necessary.

A procedure for the quantitative determination of tolbutamide in plasma is described herein which is based upon the yellow color produced by the reaction of tolbutamide with Ehrlich's reagent (*p*-N-dimethylaminobenzaldehyde) under specified conditions. Since normal plasma constituents do not interfere, a zero hour sample is not necessary. The average recovery of added tolbutamide from human plasma was 100.4 per cent with a standard deviation of ± 5.2 per cent.

From the Department of Product Research and Development, The Upjohn Company, Kalamazoo, Mich.

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*Registered trademark, The Upjohn Company, Kalamazoo, Mich.

MATERIALS AND METHODS

Reagents.—

1. Phosphoric acid: 0.46 ml. of 85 per cent 0.30 M phosphoric acid diluted to 200 ml. with distilled water.

2. Phosphoric acid: 0.46 ml. of 85 per cent 0.15 M phosphoric acid diluted to 100 ml. with distilled water.

3. Chloroform: (Mallinckrodt) analytical reagent grade chloroform sealed in glass bottles.

4. Alcohol, 95 per cent ethanol (Commercial Solvents Corporation).

5. Ehrlich's reagent solution: *p*-N-dimethylaminobenzaldehyde (Eastman Organic Chemicals). Purified by dissolving in 95 per cent ethanol, decolorizing with carbon, and crystallizing by adding water; 100 ± 1 mg. is dissolved in alcohol and diluted to 25 ml. in a brown glass bottle. The solution is prepared immediately before using.

6. Standard tolbutamide solution: A 0.7 to 0.8 Gm. quantity of the sodium salt of tolbutamide is weighed to the nearest milligram and transferred quantitatively to a 500 ml. volumetric flask and made to volume with water. Various volumes, 10 to 80 ml., of this solution are diluted to 500 ml. Sodium tolbutamide × 0.9247 = tolbutamide.

Apparatus.—

1. Separatory funnels: Sixty milliliter separatory funnels fitted with Teflon stopcock plugs were used for the extractions. After being washed with a synthetic detergent and rinsed with distilled water, they were further rinsed with alcohol. Pledgets of Pyrex glass wool, previously washed two times with alcohol, were inserted in the stems of the funnels to remove droplets of water from the chloroform phase. Prior to being used the funnels were rinsed with 10 ml. chloroform which was allowed to filter through the glass wool pledget.

2. Spectrophotometer: A Beckman Model B spectrophotometer with 1 cm. cuvettes was used for the absorbance measurements.

3. Vacuum evaporator: A vacuum evaporator constructed in these laboratories was used for the evaporation of the solvents.

Recommended Procedures.—

1. Extraction: Duplicate 1 ml. aliquots of oxalated plasma are mixed with 2 ml. of M/30 phosphoric acid in separatory funnels containing 10 ml. chloroform. The acidified plasma, pH 4.5 to 4.8,[2] is extracted with the chloroform by gently rocking the separatory funnel 20 times to and fro about an axis passing through the stopcock. The chloroform layer is slowly drained through the pledget of glass wool and collected in a 50 ml. round bottom flask which can be fitted to the vacuum evaporator. The extraction is repeated with two additional 10 ml. portions of chloroform. The combined extracts are concentrated to dryness at room temperature. Samples can be left at this stage.

2. Color development: To one of the dry residues is added 2 ml. of Ehrlich's reagent solution. To the other dry residue 2 ml. of alcohol is added. To a clean flask 2 ml. of the Ehrlich's reagent solution is added. All 3 are taken to dryness as above. (The second sample corrects for the amount of color extracted from the plasma by the chloroform, the "chloroform blank" [CB], and the third flask corrects for the amount of color due to the reagent, i.e., "reagent blank" [RB]. See paragraph on calibration below and also the Discussion.)

The dry residues are then heated for 2.5 hours in a water bath at 70° C. ± 1° C. To prevent further development of color the flasks are then removed and cooled in running tap water.

3. Spectrophotometry: Ten milliliters of alcohol is added to the dry residues, and the absorbances (optical densities) of the solutions are measured at 395 mμ with alcohol in the reference cuvette within 8 minutes after the alcohol is added.

4. Calibration: Aliquots of 1 ml. of the standard sodium tolbutamide solutions are added to plasma from a person not on tolbutamide therapy. One milliliter of M/15 phosphoric acid is added to each sample and the samples are carried through the above procedure. From the data a plot of absorbance versus concentration of tolbutamide can be prepared or alternatively the average absorptivity can be calculated. Beer's law was followed in the range 0 to 200 μg tolbutamide per milliliter of plasma; absorbances of about 0.07 to 0.70 were obtained. A positive intercept in the calibration curve results from the use of alcohol in the reference cuvette instead of the unstable reagent blank.

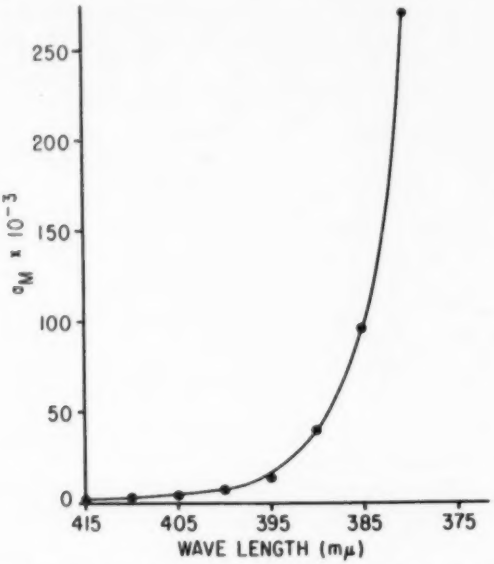

Fig. 1.—Absorption spectrum of the tolbutamide; Ehrlich's reagent chromogen (molar absorptivity calculated on the basis of the tolbutamide concentration of the final solution).

DISCUSSION

The color producing reaction between tolbutamide and Ehrlich's reagent could not be made to take place in solution although various types of solvents were used.[5] However, by heating the dry residue obtained as described reproducible results were obtained.

Fig. 1 is a plot of wave length versus molar absorptivity. Since the absorbance changes so rapidly with decreasing wave length it is important that the wave length scale of the instrument is accurate.

The color does not fade rapidly. There is no fading during the first 8 minutes. A 4 per cent fading occurred during the next 22 minutes.

The customary reagent blank, i.e., no plasma plus the reagent, is not used in the reference cuvette because this would necessitate a reagent blank for each 3 determinations as the color of the reagent blank is also unstable. The absorbance of the reagent blank was determined each time a new Ehrlich's reagent solution was made. The absorbance of the reagent blank is fairly reproducible (see Table I).

In a determination such as this the blank value of plasma (no tolbutamide present) is due to two factors. One is the color extracted from the plasma by the chloroform (the chloroform blank) and the other is the presence of materials extracted by the chloroform which react with the Ehrlich's reagent to produce color (the plasma blank). The first factor can be evaluated readily by carrying a sample through the procedure and not adding the color-producing reagent. For the evaluation of the second factor a zero hour sample is necessary.

TABLE I. EVALUATION OF BLANK VALUES

PLASMA SAMPLE	A_o PLASMA (ETC.) + EHRLICH'S	REAGENT BLANK NO PLASMA (ETC.) + EHRLICH'S	$CHCl_3$ BLANK PLASMA (ETC.) + NO EHRLICH'S		PLASMA BLANK $A_o - (RB + CB)$	
			A_{395}	TOLBUTAMIDE EQUIVALENT*	A_{395}	TOLBUTAMIDE EQUIVALENT*
ST	0.099	0.086	0.010	3 μg./ml.	0.003	1 μg./ml.
DEG	0.093	0.077	0.010	3	0.006	2
RCA†	0.089	0.078	0.005	2	0.006	2
PO	0.083	0.076	0.008	3	−0.001	0
DWA	0.084	0.075	0.002	1	0.007	2
ERB	0.082	0.073	0.001	0	0.008	2
POL	0.090	0.081	0.006	2	0.003	1
PT	0.085	0.076	0.005	2	0.004	1
CH	0.090	0.085	0.008	2	−0.003	−1
RM	0.088	0.083	0.007	2	−0.002	−1
			Average	2.3		0.9‡

*Absorbance of 0.001 = 0.3 μg tolbutamide per milliliter of plasma.
†Diabetic patient.
‡Average correction for zero hour sample.

Then the plasma blank, PB = total absorbance (A_o) − reagent blank (RB) − chloroform blank (CB). The data in Table I indicate a plasma blank equivalent to about 1 μg tolbutamide per milliliter. Since plasma levels of tolbutamide range from 30 to 200 μg per milliliter for individuals receiving the drug[1, 3, 4] the plasma blank may be ignored; thus, the need for a zero hour sample is obviated.

TABLE II. RECOVERY OF ADDED TOLBUTAMIDE FROM HUMAN PLASMA
(Micrograms per milliliter of plasma)

ADDED	FOUND	PER CENT RECOVERY			AVERAGE
83	89, 90, 81	107.2	108.4	97.5	104.5
111	115, 116, 115	103.6	104.5	103.6	103.9
138	148, 142, 135	107.2	102.9	97.8	102.6
166	173, 170, 151	104.2	102.4	91.0	99.2
194	191, 193, 187	98.5	99.5	96.4	98.1
222	210, 208, 208	94.6	93.7	93.7	94.0
Average ± standard deviation (n = 18) 100.4 ± 5.2 per cent.					

The recovery of added tolbutamide to human plasma is shown in Table II. The average recovery is 100.4 per cent with a standard deviation of 5.2 per cent. The values have been corrected for the chloroform blank but not for the plasma blank. That is, the data are comparable to the situation wherein a zero hour sample is not possible.

Hemolysis was not a problem in these studies.

SUMMARY

A procedure is described for the determination of tolbutamide in human plasma. The acidified plasma is extracted with chloroform, the chloroform extract is taken to dryness, an alcoholic solution of Ehrlich's reagent (*p*-N-dimethylaminobenzaldehyde) is added and the solution taken to dryness. The dry residue is heated at 70° C. for 2.5 hours. Alcohol is added and the absorbance of the solution is measured at 395 mμ. The average recovery of tolbutamide from human plasma has been 100.4 per cent with a standard deviation of 5.2 per cent. A zero hour sample is not necessary.

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AN IMPROVEMENT OF THE METHOD FOR DETERMINATION OF PLASMA AND URINE HEMOGLOBIN

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THE benzidine method for the measurement of small amounts of hemoglobin[1] utilizes undiluted specimens when the concentration of hemoglobin is less than 30 mg. per 100 ml. With higher concentrations, appropriate dilution of the unknown is necessary, a process which consumes time and may reduce accuracy. The precise measurement of a very small volume would permit the use of undiluted material even when the concentration of hemoglobin is relatively high. The purpose of this communication is to describe a modification of the "Dacie pipette"[2] which may be used for the measurement of volumes as small as 1 lambda. This permits the use of undiluted specimens with concentrations of hemoglobin as high as 400 mg. per 100 ml.

METHODS

The instrument consists of a Pasteur pipette used to brace a small length of capillary tubing. This tubing is broken to a desired length—to correspond to the approximate volume to be measured—and is inserted into the tip of the pipette and fixed there with a bit of modeling clay. In the rubber bulb of the pipette, a hole is made about 2 mm. in diameter (Fig. 1). The reagents used are those employed for the benzidine method: an acid solution of benzidine, hydrogen peroxide, and acetic acid.[1]

The tubing is filled by holding the pipette horizontal and touching the tip of the capillary to the surface of the liquid to be measured. This means that the test tube of liquid must be tipped to bring the liquid to the rim. The capillary is emptied by holding the pipette vertical and permitting the liquid to run into the benzidine solution. To empty it completely, the hole in the rubber bulb is covered with the finger tip, and the bulb is gently squeezed and released to empty and rinse the capillary. The hemoglobin-benzidine mixture is now treated with hydrogen peroxide in the usual manner[1] and the intensity of color which develops is measured photometrically. It is unnecessary to know the exact volume of the capillary, because the same capillary is used to measure an identical amount of a standard hemoglobin solution and the results of the standard and the unknown are compared to derive the concentration of hemoglobin in the unknown. The same capillary may be used to measure an entire series of unknowns if, after each measurement, it is rinsed first with water and then with acetone.

The standard solutions of hemoglobin are prepared first by measuring the concentration of hemoglobin in a sample of whole blood by the cyanmethemoglobin method.[3] The red cells are then hemolyzed by freezing and thawing and the blood is diluted with a mixture of saline and glycerol (equal parts) to achieve several desired concentrations: 20, 80, and 200 mg. of hemoglobin per 100 ml. provide a satisfactory covering of the concentrations measured by this method. These glycerolized preparations are stable and may be retained and used for many months.[4]

From the Department of Hematology, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D. C.
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RESULTS

Several trials were undertaken to establish the limitations of the method. Table I demonstrates the reproducibility of results when two pipettes were used to measure repeatedly a single sample of plasma. There is less than 1 per cent variability. Table II demonstrates the results of a "blind" experiment. A sample of plasma was stained by adding to it a small amount of gentian violet so that the operator could not estimate by eye the hemoglobin concentration.

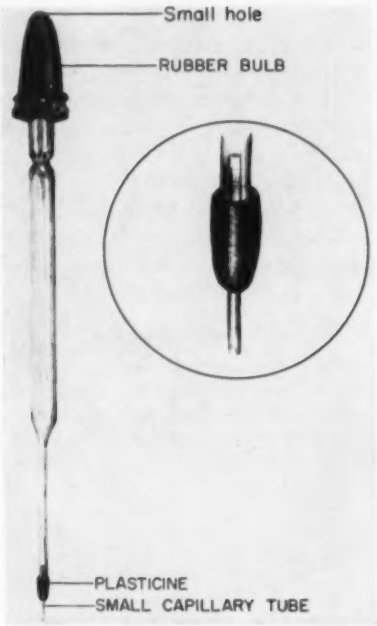

Fig. 1.—A modification of the Dacie pipette to enable measurement of small volumes.

Then the sample was divided into 6 tubes and a small amount of hemoglobin was added to each, measuring it to achieve concentrations of approximately 40, 60, 80, 120, and 200 mg. hemoglobin per 100 ml. The concentration of hemoglobin of each specimen was measured on 3 different days and the results are shown in Table II. In each instance the results agree within about 5 per cent.

It is possible to encompass the entire range of plasma hemoglobins using 3 or 4 of these little pippettes, each a different size. Table III demonstrates how this was done with 3 pipettes, the approximate volumes of which were 20, 5, and 1.5 lambda. For any given unknown the proper pipette is selected so that the reading of transmittance on the photometer scale lies within the range of best accuracy of the instrument, which is usually between 20 and 80 per cent.

TABLE I. REPLICATE DETERMINATIONS OF PLASMA HEMOGLOBIN PERFORMED ON A SAMPLE OF PLASMA USING TWO PIPETTES

TUBE	PHOTOMETER READING	MG./100 ML.	TUBE	PHOTOMETER READING	MG./100 ML.
1	28[3]	87.2	1	31	87.0
2	29	87.0	2	31	87.0
3	29	87.0	3	31	87.0
4	29	87.0	4	31	87.0
5	28[3]	87.2	5	31	87.0
6	28[3]	87.2	6	31	87.0
7	29	87.0	7	31	87.0
8	29	87.0	8	31	87.0
9	29	87.0	9	31	87.0
10	28[3]	87.2	10	31[2]	86.8
11	29	87.0	11	30[3]	87.5
12	29	87.0	12	31	87.0
13	29	87.0	13	31	87.0
14	29	87.0	14	31	87.0
15	29	87.0	15	31[2]	86.8
Standard	56	40.0	Standard	58[2]	40.0

TABLE II. "BLIND" DETERMINATION OF PLASMA HEMOGLOBIN IN STAINED PLASMAS CONTAINING APPROXIMATELY 40, 60, 80, 120, 160, AND 200 MG. PER 100 ML.

	TUBE	MG. PER CENT		TUBE	MG. PER CENT		TUBE	MG. PER CENT
Nov. 18	1	39	Nov. 20	1	38	Nov. 25	1	42
	2	66		2	62		2	57
	3	86		3	90		3	90
	4	114		4	115		4	118
	5	168		5	173		5	173
	6	201		6	201		6	201

TABLE III. DEMONSTRATING THE USE OF PIPETTES OF SEVERAL SIZES TO PERMIT MEASUREMENT OF A WIDE RANGE OF PLASMA HEMOGLOBIN CONCENTRATIONS

PIPETTE 1 APPROXIMATE VOLUME 20 LAMBDA		PIPETTE 2 APPROXIMATE VOLUME 5 LAMBDA		PIPETTE 3 APPROXIMATE VOLUME 1.5 LAMBDA	
PLASMA Hb CONCENTRATION (MG./100 ML.)	PHOTOMETER READING	PLASMA Hb CONCENTRATION (MG./100 ML.)	PHOTOMETER READING	PLASMA Hb CONCENTRATION (MG./100 ML.)	PHOTOMETER READING
0	0	20	79	80	76
10	54	40	57	100	65
20	32	60	44	120	64
30	18	80	35	160	58
--	--	100	27	200	52
--	--	120	21	300	33
--	--	--	--	350	29
--	--	--	--	400	25
--	--	--	--	450	21

Occasionally a specimen may be encountered where the concentration of hemoglobin exceeds 400 mg. Such specimens may be diluted and measured by the benzidine method or, better, the concentration of hemoglobin may be measured directly by the cyanmethemoglobin technique,[3] taking care to add a sufficiently large volume of the unknown and correcting the results accordingly.

SUMMARY

A modification of the "Dacie pipette" is described which permits the measurement of small volumes of plasma or urine. This permits the use of the

benzidine method for microhemoglobinometry without preliminary dilution of the unknowns. The use of these pipettes shortens the time required for the performance of the test and, by eliminating the measurements required for the dilutions, probably improves the accuracy of the method.

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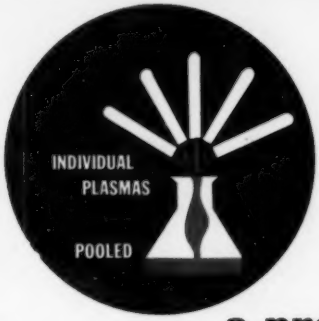
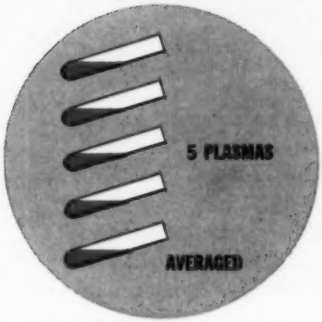
Erratum

In the article by Ralph V. Ford, M.D., and J. B. Rochelle III, M.D., entitled "The Differing Mechanisms of Action of Mercurials, Carbonic Anhydrase Inhibitors, and Chlorothiazide as Diuretic Agents," which appeared in the January issue of the JOURNAL, pages 53-63, Fig. 3 (p. 57) and Fig. 4 (p. 58) should be transposed. The legends, however, are in their correct position.



a prothrombin time control:

can be..., should be..., and why.



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PATHOLOGY SEMINARS

Edited by ROBERT S. HAUKOHL, M.S., M.D., F.C.A.P., Assistant Professor of Pathology, Marquette University School of Medicine and W. A. D. ANDERSON, M.A., M.D., F.A.C.P., F.C.A.P., Professor of Pathology and Chairman of the Department of Pathology, University of Miami School of Medicine. Written by 5 contributors.

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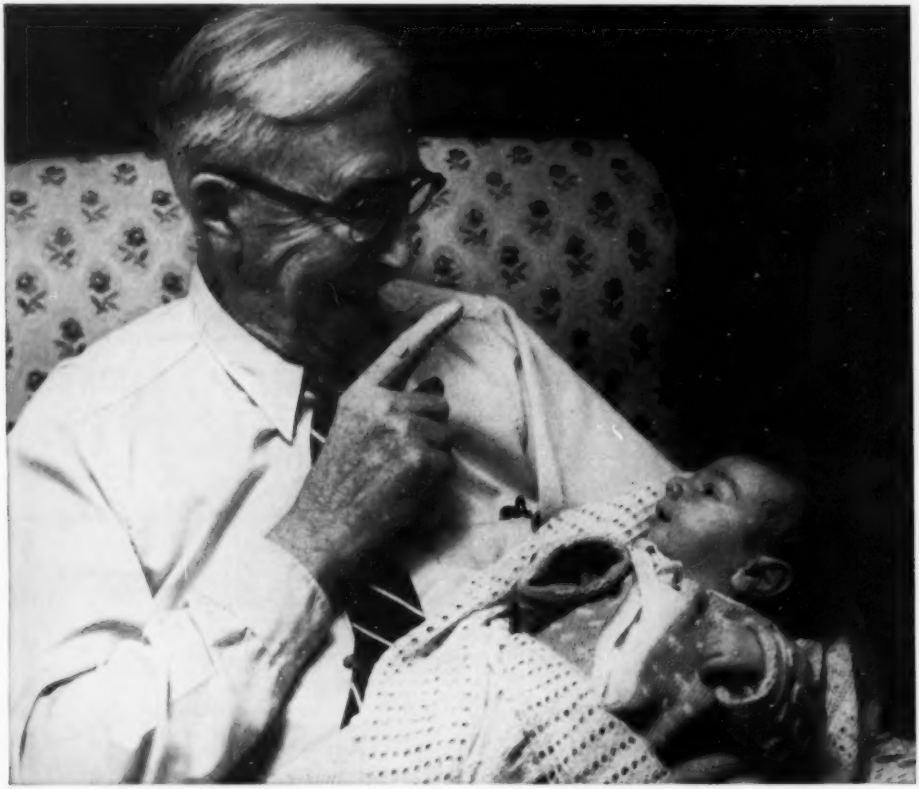

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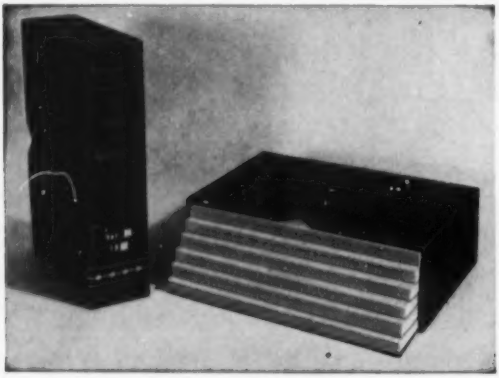

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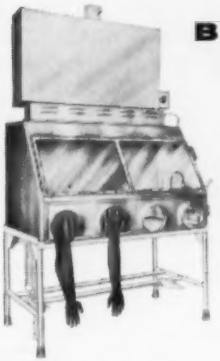

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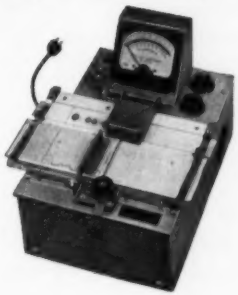

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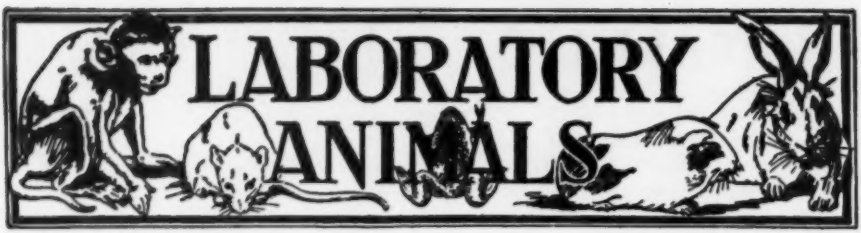

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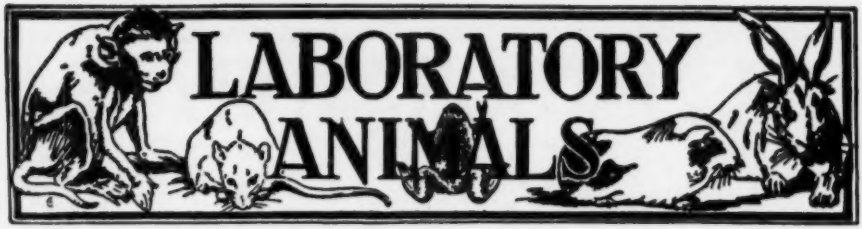

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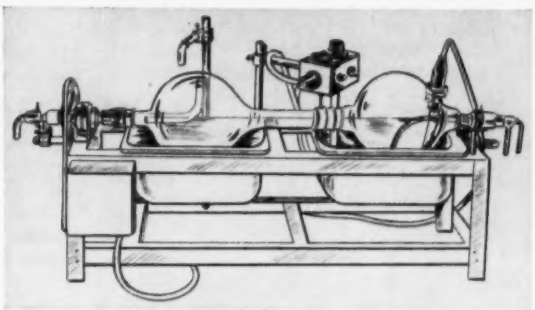

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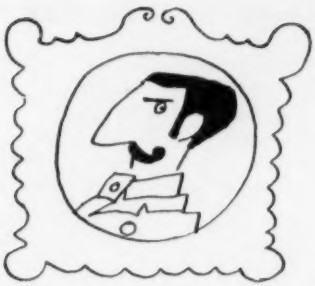

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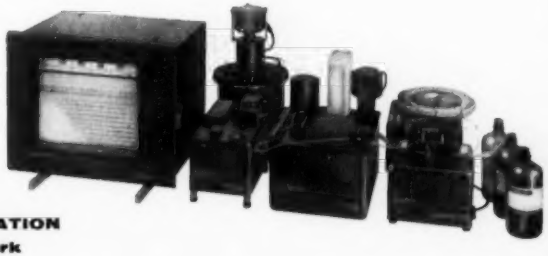

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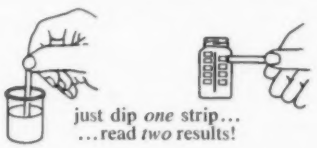

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